

**STUDI KLINIS, SITOGENETIKA DAN MOLEKULER
PADA INDIVIDU DENGAN RETARDASI MENTAL DI
SEMARANG**

***CYTOGENETICS, MOLECULAR AND CLINICAL
STUDIES AMONG MENTALLY RETARDED
INDIVIDUALS IN SEMARANG***



Thesis

**Submitted to fulfill the assignment and fit-out requisite
in passing Post-graduate Program Majoring Genetics Counseling
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Master of Biomedical Sciences

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DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement is made in the text

Semarang, Desember 2008

Farmaditya EP Mundhofir

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ABBREVIATIONS

AAMR	American Association on Mental Retardation
AS	Angelman Syndrome
bp	base pair
dCTP	deoxycytidine triphosphate
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ECARUCA	European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations
EDTA	Ethylene diamine tetra-acetic acid
FBS	Fetal Bovine Serum
FISH	Fluorescence in situ hybridization
FXS	Fragile-X Syndrome
FXTAS	Fragile-X associated Tremor Ataxia Syndrome
IQ	Intelligence Quotient
MB	mega base
MEM	Minimum Essential Medium
MLPA	Multiplex Ligation dependent Probe Amplification
MR	Mental retardation
MRCH	MRC-Holland
PAR	Pseudo autosomal region
PBS	phosphate buffered saline
PCR	polymerase chain reaction
POF	Premature ovarian failure
PW /AS	Prader-Willi/Angelman Syndrome
PWS	Prader-Willi Syndrome
RUNMC	Radboud University Nijmegen Medical Centre
SDS	sodium dodecyl sulphate
SLB	Sekolah Luar Biasa (special school)
SSC	standart saline citrate
STDs	Sub-telomeric deletions and duplications
Taq	Thermus Aquaticus
TBE	Tris Borate EDTA
TE	Tris EDTA
WHS	Wolf-Hirschorn Syndrome
WHSCR	Wolf-Hirschorn Syndrome Critical Region
XLMR	X -Linked mental retardation

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ABSTRACT

Background: Mental retardation (MR) is still a major health problem worldwide. Genetic factors play a significant role in MR. In Indonesia, only a few genetic studies have been performed in the MR population so far.

Objective: To identify major genetic causes of mental retardation, excluding Down Syndrome, in Semarang Jawa-Tengah Indonesia, including cytogenetics, Fragile-X Syndrome, Sub-telomeric deletions and duplications (STDs), Prader-Willi Syndrome (PWS) and Angelman Syndrome (AS). In addition, to establish a MR diagnosis protocol for future studies that will be conducted in the Indonesian population.

Methods: A total of 122 mentally retarded pupils from three special schools were screened for cytogenetic abnormalities and CGG repeats in the *FMR-1* gene. Subsequently, they were screened for STDs and for PWS/AS using Multiplex Ligation-dependent Probe Amplification (MLPA) analysis. All pupils were clinically examined following an adapted diagnostic protocol from the RUNMC. The members of one family of an affected male pupil with a full CGG repeat expansion of the *FMR-1* gene were also screened for CGG repeats and Southern Blot analysis were performed.

Results: A full CGG repeat expansion was identified in one subject and a mosaic pattern of expansions in the premutation range and full mutation was identified in another subject respectively. In the 1 Fragile-X family showed two premutation female carriers, two males with mosaic pattern in the premutation range and one female with a full mutation. Sub-telomeric deletions and/or duplications were identified in 13 samples. Sub-telomeric deletions and duplications results are discussed. None of the selected samples for PWS/AS analysis were positive.

Conclusions: This study showed that several genetic factors contribute to and play a role in the development of MR in Indonesia. A standardized MR clinical examination protocol, adapted from the RUNMC, can be used for further research in Indonesia.

ABSTRAK

Latar Belakang:

Retardasi mental masih merupakan masalah kesehatan dunia termasuk di Negara-negara maju. Faktor genetik berperan secara signifikan sebagai penyebab retardasi mental. Sampai saat ini, baru sedikit penelitian genetik pada populasi retardasi mental di Indonesia.

Metode:

Skrining abnormalitas sitogenetik dan pengulangan CGG pada gen FMR-1 telah dilakukan pada 122 murid SLB. Selanjutnya pada populasi tersebut dilakukan skrining untuk mikro duplikasi dan mikro delesi sub telomerik dan untuk sindrom Prader-Willi/Angelman menggunakan teknik MLPA. Pemeriksaan fisik menggunakan protokol diagnostik yang diadaptasi dari RUNMC telah dilakukan pada semua siswa. Anggota keluarga penderita Fragile-X juga telah diskriminasi untuk pengulangan CGG dan analisis Southern Blotting.

Hasil:

Satu siswa teridentifikasi dengan Full mutasi dan satu siswa lainnya teridentifikasi memiliki mosaik antara full mutasi dengan premutasi. Pada keluarga dengan Fragile-X, dua wanita merupakan karier premutasi, dua pria mempunyai pola mosaik dalam batas premutasi. Delesi dan duplikasi subtelomerik telah teridentifikasi pada 13 siswa. Hasil delesi dan duplikasi sub-telomerik ini didiskusikan. Dari sampel yang dipilih untuk pemeriksaan PWS/AS tidak satupun ditemukan hasil positif.

Kesimpulan:

Pada penelitian ini ditunjukkan bahwa beberapa faktor genetik berkontribusi dan mempunyai peran sebagai penyebab RM di Indonesia. Protokol pemeriksaan klinis yang diadaptasi dari RUNMC bisa digunakan untuk penelitian selanjutnya di Indonesia.

CHAPTER I

INTRODUCTION

I.1. Background

Mental retardation (MR) is still a major health problem in all countries including the developed countries. Besides the fact that educational and psychological aspects regarding MR need more attention, some cases of severe MR require nursery, guidance, and surveillance as long as they live¹. Some etiologies of MR are known, like biochemical causes, chromosomal abnormalities, mutations of single gene (Mendelian disorders/mitochondrial disorders), multi-factorial disorders or because of environmental factors such as toxins, infections, and trauma. However, genetic factors have a crucial role since approximately half of MR cases have a familial history².

About 38.545 Pupils have been registered in special schools for mentally retarded for the whole of Indonesia³, but this does not represent the total number of MR pupils in Indonesia. Factors such as lack of awareness, parents' educational background, availability of special schools (in some areas), and economic background could be the few reasons why children with MR are not formally schooled. Semarang is the capital of Central Java province with total residents of 1.389.416⁴. Most residents in this city include Javanese, Chinese and other ethnic groups in a small percentage. Semarang has 8 special schools for MR children with a total number of 942 pupils³. Indonesia is a developing country, where health insurance is not at all mandatory for all the citizens making mentally retarded children really a big problem for the family both financially and morally. Therefore, early diagnosis and prevention of MR should be one of the top health priorities.

The knowledge of genetic factors of mental retardation by cytogenetics, molecular and clinical assessment is an advantage in giving early diagnosis and prevention through genetic counseling to subject's family. These serve as diagnostics tool in determining genetic factor that may play role as etiological cause of MR. Nowadays, the above mentioned assessments could well be done in Indonesia. But due to lack of facilities in Indonesia, further molecular assessments have to be carried out in other centre abroad.

Up till now there are only few studies on MR in Indonesian population carried out by Indonesian researchers or in collaboration with researchers abroad. No MR protocol has been applied for diagnosis of MR in Indonesia, thus this study aims to establish MR protocol for diagnosis in Indonesia. More over genetic assessments as an etiological diagnostic tool for MR have not yet been recognized as a routine diagnostic tool in Indonesia. The procedures for genetics assessment will be the main focus of this research in order to apply this MR protocol for diagnosis in Indonesia.

I.2. Research Questions

1.2.1 General Research Question

What is the percentage of genetic factors playing a role in the etiology of mental retardation in Indonesian Population?

1.2.2 Research question in detail

1. What are the genetic factors that causing MR in Semarang?
2. Is there any clinical phenotype genotype association resulted by cytogenetics and molecular analysis?

1.3. Research purposes

1.3.1 General research purposes

The purposes of this research include:

1. To identify major genetic causes of mental retardation in Semarang, Jawa-Tengah Indonesia.
2. Establishing MR diagnosis protocol for Indonesian subjects is one of the major importances of this research.

1.3.2. Specific research purposes

1. To determine if genetic factors are responsible for mental retardation in Semarang
2. To find out the frequency of chromosomal abnormalities excluding Down Syndrome in special schools in Semarang.
3. To asses and evaluate the implementation of diagnostic protocols been employed in the developed countries.

1.4. Research advantages:

1. To encourage public awareness of genetic diseases. Infectious diseases are still top concern for government and medical organizations. Therefore starting a large population based genetic survey in Indonesia will possibly increase this public awareness. For stakeholders it is a very important concern to provide awareness about the role of genetics for such a major health problem like mental retardation.
2. To lay down a basis for genetic counseling. Genetic counseling is not that common in Indonesia, though with increasing public awareness in genetic

diseases there will be an increasing demand for genetic counseling. Genetic counselors should be able to recognize genetic heterogeneity with geographic and ethnic differences. The results of this research should become a basis for genetic counselors when giving counseling to the parents.

1.5. Research Originality

1. Screening on Fragile-X and cytogenetic in individual with Mental Retardation in Indonesia have been performed initially by Faradz et al ⁵⁻⁷.
2. Screening on PW/AS and STDs in individual with Mental Retardation in Indonesia is the first study in Indonesia.

CHAPTER II

LITERATURE REVIEW

II.1 Mental Retardation

II.1.1 Definiton and Etiology

Mental retardation (MR) refers to substantial limitations in present functioning. It is characterized by significant subaverage intellectual functioning i.e $IQ < 70$, existing concurrently with related limitations in two or more of the following applicable adaptive skill areas: communication, self-care, home living, social skills, community use, self-direction, health and safety, functional academics, leisure and work. The age of onset should be before age 18⁸.

Many environmental and genetic factors can cause MR like biochemical causes, chromosomal abnormalities, mutations of single gene (Mendelian disorders/mitochondrial disorders), multi-factorial disorders or because of environmental factors such as toxins, infections, and trauma⁹. Nevertheless, genetic factors have a crucial role since approximately half of MR cases have a familial history².

II.1.2. Genetic Causes

Several genetic causes of MR are known, most common are the large microscopic numerical and structural cytogenetic abnormalities (> 4 MB), such as trisomy 21 (Down syndrome), large deletions and duplications and unbalanced reciprocal and Robertsonian translocations¹⁰. Down syndrome is the best known chromosomal abnormality as cause of MR with the highest prevalence. This abnormality occurs due to meiotic non-disjunction, mostly maternal with a result an

extra 21 chromosome in the child. With routine chromosomal analysis, trisomies like trisomy 21 can be easily detected¹⁰.

Individuals with MR, dysmorphic features with or without positive family history (usually without positive family history) and without microscopic chromosomal abnormalities can be due to cryptic chromosomal rearrangements like sub-microscopic sub-telomeric deletions or duplications. This has been identified as another common cause of MR^{11,12}.

Chromosome ends (telomeres) consist of TG-rich hexamere (TTAGGG)_n and repeated several thousand times. Next to the telomeres there is the sub-telomeric region located; consisting of complex families of repetitive DNA sequences¹³. These telomeric regions are the highest gene concentrations in the human genome and extremely gene rich¹⁴. Abnormalities in this region are associated with severe phenotypic consequences and estimated to account for approximately 5% of mental retardation¹¹. Some studies by Knight et al, Koolen et al, Rooms et al and Nothrop et al. has led to awareness that subtelomeric deletions below the level of light microscopes are significant cause of mental retardation^{12,15-17}.

Penrose in 1938 was first to observe that more males than females in the population are mentally retarded in a survey and classification of those in institutional care and their relatives. In 1970's Lehrke was first to suggest that there may be genes coding for intellectual function located in X chromosome¹⁸. This hypothesis has been substantiated by numerous subsequent studies in many countries and an approximately 30% excess of males being affected with mental retardation¹⁹⁻²¹. As illustrated in Table 1, excess of male pupils are also shown in Semarang with male-to-female ratio was 1,31. The excess male pupils than female pupil in our population were observed to be similar with previous studies above.

Table 1. Gender distribution of pupils in Semarang special school term 2005/2006

No.	School Name	Male Pupils	Female Pupils	Total Pupils
1.	SLB YPAC Semarang	95	77	162
2.	SLB Swadaya	26	27	53
3.	SLB Widya Bhakti	121	95	216
4.	SLB Pelita Ilmu	24	12	36
5.	SLB Hj. Sumiyati	30	18	48
6.	SLB Dharma Mulia	29	26	55
7.	SLB Immanuel	14	4	18
8.	SLB Negri Semarang	26	19	45
Total		365	278	643

Data were taken from each school at preliminary study.

X-Linked Mental Retardation (XLMR) can be generally classified into two categories; syndromic XLMR (S-XLMR) that are associated with a specific or characteristic phenotype and non-syndromic XLMR (NS-XLMR) that do not present with consistent clinical features²². Non-syndromic XLMR was thought to be more frequent than the syndromic XLMR conditions^{21,23}. Nevertheless, due to the development of molecular techniques which is used to classify family and better detailed examination in the patient, the proportion of S-XLMR will be increase simultaneously with the decrease of NS-XLMR²².

Fragile-X syndrome is one of the XLMR and become the most common inherited abnormality causing MR²⁴. This syndrome is commonly seen in males. However, females can also be affected. Approximately 50-60% of female full mutation carriers will have borderline to mild mental retardation²⁵. The Fragile X syndrome is due to a mutation in a gene on the X chromosome leading to hyper-expansion of a trinucleotide (CGG) repeat sequence. There is a repetitive CGG sequence on the 5'UTR of the *FMRI* gene and normal individuals have approximately 5 to 44 CGG repeats. There is a gray zone of 45 to 54 repeats that can be associated with minor instability from generation to generation. The premutation is defined as 55

to 200 repeats and the full mutation of the *FMRI* gene involves an expansion of >200 CGG repeats and is clinically associated with features of Fragile-X syndrome²⁶⁻²⁷.

The diagnostics for Fragile-X syndrome is principally based on Southern blotting (DNA digested with specific restriction endonucleases) or on direct amplification of the CGG-repeat using flanking primers. Using standard PCR amplification, alleles that have repeats above 120 CGGs are difficult to detect, but PCR amplification is needed to obtain accurate sizing of permutations^{26,28}. This is crucial to calculate the risk of having affected children for carrier females, and to distinguish intermediate/gray zone alleles from permutations^{25,28}.

Panagopoulos et al. and Pena et al reported a possibility to diagnose FXS using Methylation Specific PCR (MS-PCR) of the FMR-1 locus. This technique is promising a possibility to amplify bigger size of fragment which was not possible to amplify with regular PCR before^{29,30}. Nevertheless, this technique had a limitation. Although the method can be used to analyze female individuals, there is an additional problem due to the methylation of the inactive X chromosome³⁰.

There are other methods to detect FXS. Willemsen et al invented a non-invasive technique to detect FXS based on FMRP specific antibody in the hair root³¹. Recently, Tassone et al invented a rapid polymerase chain reaction (PCR)-based screening tool for expanded *FMRI* alleles. This method uses a chimeric PCR primer that targets randomly within the expanded CGG region³².

Prader-Willi and Angelman syndrome are genetic disorders that show different levels of mental retardation. Prader-Willi syndrome (PWS) features mild to moderate mental retardation and Angelman syndrome (AS) severe mental retardation^{32,33}. These syndromes can occur due to absence of the paternally or maternally derived chromosome 15q11-13 region by several genetic mechanisms.

When there is absence of paternal chromosome 15q11-13 region it will manifest as Prader-Willi Syndrome, while absence of the maternal chromosome 15q11-13 region will manifest as Angelman syndrome³⁴⁻³⁶.

High-resolution chromosome studies at the 650-band level and in particular fluorescence in situ hybridization (FISH) testing could detect individuals with PWS and AS, because approximately 70% individuals with PWS and AS have a deletion of one chromosome 15 involving bands 15q11-q13. Whereas in PWS uniparental maternal disomy occurs in about 25% of cases, in AS uniparental paternal disomy occurs in only 1% of cases. Both syndromes and in particular AS can also be caused by mutations in imprinted genes with potentially high recurrence risks³⁷.

II.1.3 Genetic diagnosis

Genetics diagnosis for MR individual is based on dysmorpological and laboratory assesement. Most known genetics syndrome in MR is associated with specific features¹⁰. Laboratory assessment is considered as the tool to confirm the genetics diagnosis based on clinical examination. Nevertheless, some genetics etiology could not be diagnosed with dysmorphological assessment¹⁰. Thus, laboratory assesement is needed^{10,12}. However, dysmorphological assessment is the most important thing to do before laboratory and other assessment.

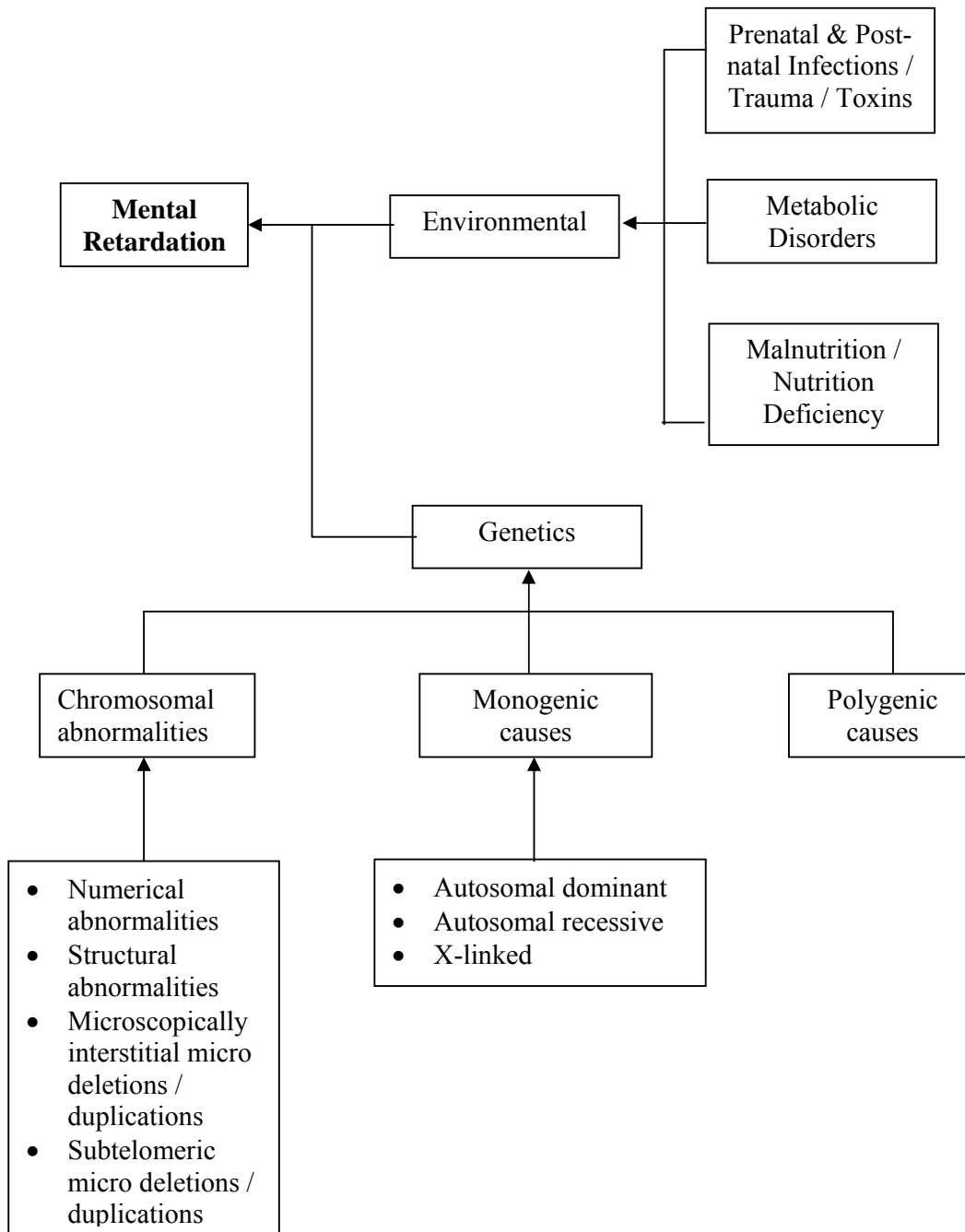
Chromosomal analysis is the most valuable analysis to reveal genetics cause of MR. With routine chromosomal analysis, numerical abnormalities such as trisomy 13,18, and 21 or monosomy such as monosomy X (45,X/Turner syndrome) can be easily detected¹⁰. Furthermore chromosomal analisys can reveal some structural chromosome abnormality such as deletion / duplication (> 4 Mb), insertion, derivation, and translocation. That is why; we do the chromosome analysis as the first laboratory assessment for the patient.

Since Fragile-X syndrome is considered as the most common inherited abnormality causing MR²⁴. Laboratory analysis for the Fragile-X syndrome is the most significant assessment after chromosome analysis. Although fragile site in the Fragile-X syndrome is detected in chromosomal analysis, nevertheless subject with permutation or mosaic permutation – full mutation of Fragile-X syndrome might not detected by routine chromosome analysis. That is why; we have to do confirmation analysis with molecular assessment.

Subtelomeric deletions and duplications (< 4Mb) are not visible by microscope¹². Thus molecular analysis of this abnormality is required. Based on the high incidence of this abnormality in MR individual^{11,12,15-17}, molecular screening of this abnormality is very important if the chromosome analysis and Fragile-X analysis are fail to detect the genetic cause of MR.

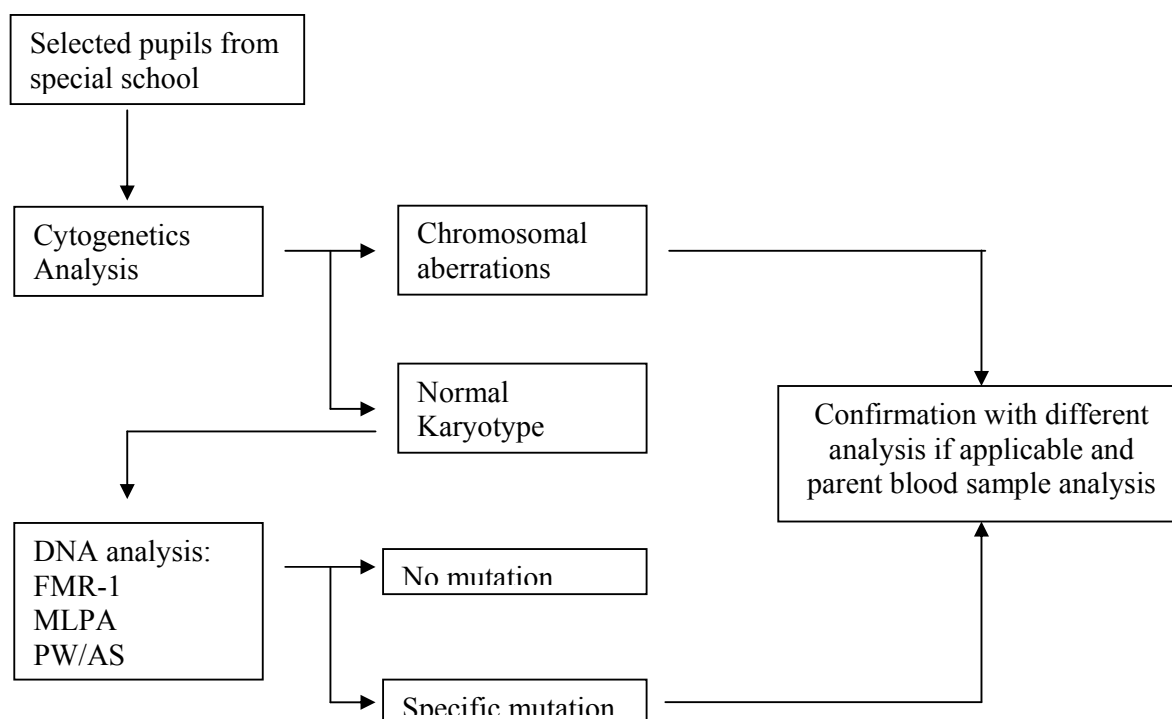
PW/AS analysis could be performed to the MR individual with suspicion of this abnormality. Since there are some genetics diseases have similar features with PW/AS syndrome, this analysis is valuable to verify genetic diagnosis of MR especially to the MR individuals with suspicion of this syndrome³⁷.

II.2. Theoretical Scheme

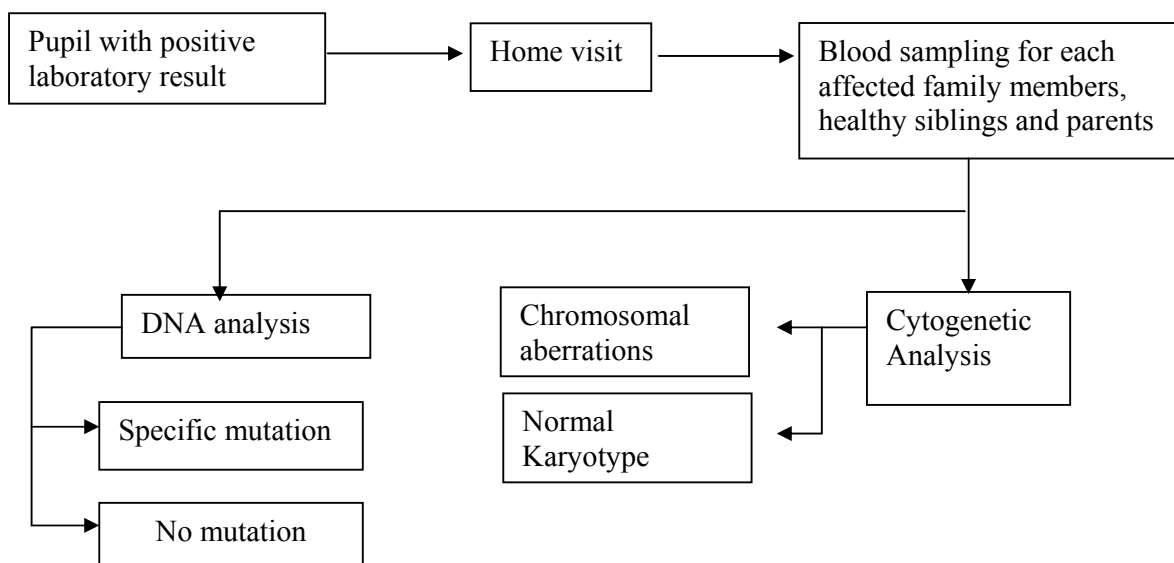


II.3. Conceptual Frame

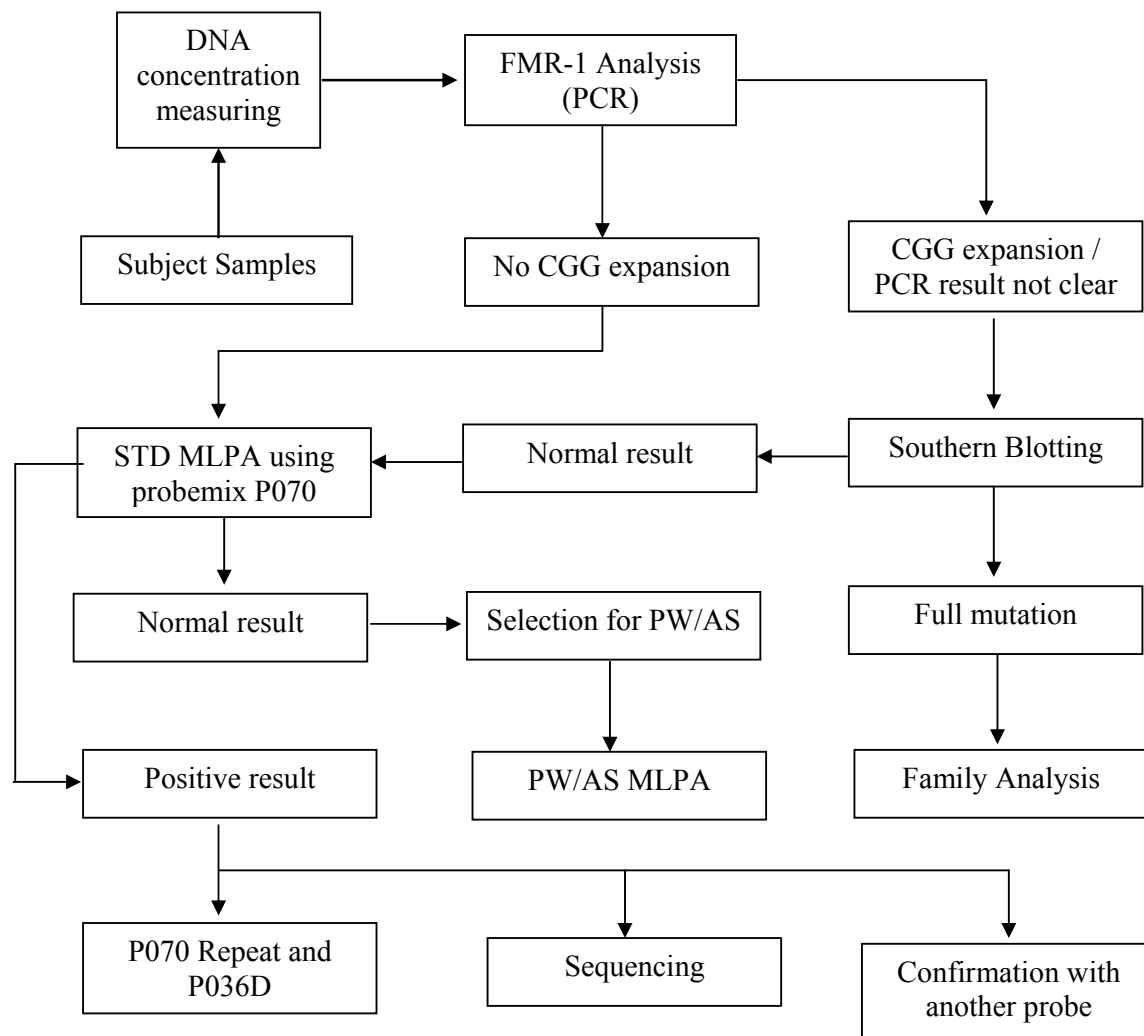
II.3.1. First screening steps



II.3.2. MR Pupil with positive laboratory results



II.3.3 Molecular analysis scheme



CHAPTER III

RESEARCH METHODOLOGY

III.1. Research aspects

III.1.1 Research field

This research project is in the field of human genetics particularly cytogenetics and molecular genetics inter-correlated with pediatrics and clinical genetics.

III.1.2 Research location

Pupils have been examined from several special schools for the mentally retarded in Semarang namely SLB Negeri, SLB Pelita Ilmu, and SLB Hj. Soemijati. Routine chromosome analysis was performed in Molecular and Cytogenetic laboratory of Center for Biomedical Research, Faculty of Medicine Diponegoro University Semarang. DNA analysis of the Fragile-X syndrome, Prader-Willi/Angelman syndrome and sub-telomeric deletions and deletions (STDs Analysis) with MLPA (Multiplex Ligation Probe Amplification) were performed in the Radboud University Nijmegen (Laboratory for DNA diagnostics, RUNMC).

III.1.3. Research period

This research conducted within 1.5 year with sample collection in the 1st semester continued with Cytogenetic preparation and DNA extraction. In 2nd and 3rd semester for molecular analysis was carried out in Nijmegen.

III.1.4 Research design

This study is a observational survey.

III.2 Material

III.2.1 Population

Mentally retarded pupils of several special schools in Semarang (SLB Hj. Soemijati, SLB Pelita Ilmu, and SLB Negeri) were included in this study.

III.2.2. Samples

Samples were taken from 122 mentally retarded pupils with an average age between 6 and 25 years (Table 4). The parents were also involved in order to obtain the family history. Furthermore, samples of parents, of pupils with a proven genetic abnormality, were taken in order to determine *de novo* or inherited occurrence. Not all parents were available for sampling.

Table 4. Total amount samples obtained from special schools in Semarang

School Name	Males	Females	Total
Hj. Soemijati	23	14	37
Pelita Ilmu	19	5	24
Negeri	52	9	61
Total	94	28	122

III.2.2.1 Inclusion criteria

Mentally retarded pupils (clinically diagnosed MR with AAMR criteria) from three special schools, whose parents signed the informed consent form, were included in this research project.

III.2.2.2 Exclusion criteria

Pupils with a known exogenic cause, such as pre-, ante- and postnatal traumas, or a history of asphyxia, non cooperative pupils and children of parents who declined to sign the informed consent form, were excluded from research participation. Also subjects with a clinical suspicion of Down Syndrome.

III.2.2.3 Subject selection

The individuals were selected based on the medical records obtained from the schools.

III.2.2.4. Clinical examination

Pupils were examined physically by thorough clinical examination using a protocol adapted from the RUNMC. Subsequently photographs were taken from all examined pupils.

III.2.2.5 Sample collection

After clinical examination 5 mL heparinized blood (for chromosome analysis) and 5-10 mL EDTA blood (for DNA analysis) were drawn from each individual.

III.2.2.6 Minimum sample required

Research with single samples in a large population has never been performed in Indonesia before; therefore we used the following formula to determine the minimum required amount of samples³⁸

$$n = \frac{Z_{\alpha}^2 PQ}{d^2}$$

$$P=0,50; Z_{\alpha}= 1,96; d=0,10$$

$$n = \frac{(1,96)^2 \times 0,50 \times (1-0,50)}{(0,10)^2} = 96$$

III.3. Methods

III.3.1 General

Parents of pupils who met our selection criteria were asked to join the research project by signing written informed consent forms. Blood samples were drawn from the individuals as described in section III.2.2.5. Parents were also informed that, if an abnormality was found in their child, they would be requested for blood sampling.

III.3.2. Laboratory Methods

III.3.2.1 Chromosome analysis

Chromosome preparation was made by culturing 10 drops of heparinized blood into two different 5 ml media (TC199 and MEM), each supplemented with 5 % Fetal Bovine Serum (FBS) and 0,025 mL Phytohemagglutinin-P (Gibco) in 37°C for 72 hours. In a MEM media tube 0,1 mL thymidine (final concentration of 0,3 µg/mL) and 3 drops of colchicine

(concentration of 1 $\mu\text{g/mL}$) were added and incubated for 24 hours and 25 minutes before cell harvesting. A TC199 tube was treated with colchicine (concentration of 1 $\mu\text{g/mL}$) but without thymidine. After the culture process was finished, the culture tube was centrifuged at 1000 rpm for 10 minutes and the supernatant was removed. A warm (37°C) hypotonic solution KCl 0,075M was added to the cell pellet and then resuspended until homogeneous and subsequently incubated at 37°C in a waterbath for 15-30 minutes. Thereafter the cell suspension was centrifuged at 1000 rpm for 10 minutes, the supernatant was removed and 5 mL Carnoy's solution (3:1 methanol: acetic acid glacial) was added slowly through the tube wall, and shaken well. These steps were repeated constantly until a clear precipitation was obtained. After a clear precipitation was obtained, then fresh Carnoy's solution was added to suspended residues. Subsequently, two drops of cell suspension were released onto a glass slide from a height of around 20 cm^{39} .

Finally, the slides were stored for approximately 3 days. After that, the aged slides were rinsed with water and put into warm Hanks solution (37°C), put into trypsin 0,1% (in warm Hanks buffer) for 10-25 seconds, depending on the sensitivity and slide age, and again rinsed with water. Thereafter the slide was flooded with Giemsa 10 % staining in buffer phosphate PH 6,8 for 1 minute (for GTG Banding staining).

III.3.2.2. DNA isolation

DNA was isolated with a salt saturation method:

EDTA frozen blood was transferred into a 50 mL tube. NH₄Cl 5-10 ml lysis buffer was added to the tube and incubated for 10 – 30 minutes at room temperature. Then the tube was centrifuged for 5 minutes at 3000-3500 RPM, the supernatant was removed and NH₄Cl lysis buffer was added again. These steps were repeated three times. Two milliliters of TE lysis buffer, Proteinase-K 10mg/mL and 100 ul 10% SDS were added and mixed gently into a white pellet and then incubated at 50°C for 24 hours. Subsequently NaCl 6M approximately one third volume of the tube was added to the suspension and centrifuged at 4000 RPM for 10 minutes. New tubes were used for the supernatant and absolute ethanol twice volume supernatant was added. DNA that looked like white substance was removed by fine needle. After that, DNA was rinsed with 70 % ethanol and transferred into a 1,5 ml tube. Excess ethanol was evaporated by leaving the tube open for at least 1 hour. Then the DNA was dissolved into TE buffer^{39,40}.

III.3.2.3.1. *FMR-I* gene amplification

Brief method description:

CGG repeats in *FMR-I* were amplified with PCR technique. Subsequently PCR products were electrophoresed in agarose gel together with specific marker to determine the length of amplified DNA⁴¹

Procedures:

Approximately 100 nanogram DNA solution was amplified in a final volume of 20µl mix solution containing 2µl 10x PFX buffer, 0,6 µl MgSO₄, 0,5µl dNTP's, 8,0 µl PCR enhancer solution, 0,6 µL forward (**gct cag etc cgt ttc ggt ttc act tcc ggt**) and reverse primer (**agc ccc gca ctt cca cca cca gct cct cca**) FAM labelled⁴², and 0,3 uL Platinum Taq enzyme and 6,4 milliQ. The samples were denatured initially for 5 min at 95°C followed by 31 cycles of 95°C for 15 seconds, 64°C for 2 min; 72°C for 2 min with a final extension of 7 min at 72°C. Thirty uL Ethyidium bromide 0,5% was added to the 300 ml agarose gel in and 60 uL was added to the TBE buffer. PCR products were electrophoresed in a 2 % agarose gel at 120 Volt for 3 hours with a 100 bp marker. A picture of the electrophoresed gel was visualized with IMAGO UV image processing. A normal CGG repeat will be between 3-55 repeats. Male samples that not produced PCR product were repeated two times. Southern Blot Analysis were performed on those samples that still not produced PCR product (after repeated two times), samples with high CGG repeat and woman with single allele (can be homozygous samples or normal and expanded allele)

III.3.2.3.2 Fragment Length Analysis for *FMR-1* gene (genescan)

Brief method description:

Fragment length analysis was used to determine the length of CGG repeat accurately. This method can be used to detect fluorescent labeled DNA fragments based on its length precisely.

Procedures:

Reverse primer with fluorescent dye (FAM) was used in the PCR process. PCR products were mixed with LIZ 500 size standard marker and formamide and subsequently sent to the ABI 3730 machine to measure the CGG repeats. One µl PCR product was mixed in a final volume of 10 uL mix solution containing 8,7 uL formamide and 0,3 uL LIZ 500 standard size marker. Raw data from ABI 3730 were analyzed using Genemapper Software version 4.0 (Apache Software). Fragment length analysis was performed with known CGG fragments both in normal control and premutation control.

III.3.2.3.3 Southern Blot Analysis**Brief method description:**

Southern blot was used to confirm the CGG results of male samples, without an *FMR-1* PCR and female samples that only showed a single allele (homozygote samples or normal and expanded allele).

DNA was digested using double digestion method for methylation studies with *HindIII* and *Eag I* restriction endonuclease enzyme to demonstrate methylation pattern.

Normal fragment size will be 2,8 Kb for *EagI* and 5,2 Kb for *HindIII* respectively. Recognition site for *HindIII* is (5' A*AGCTT; 3' TTCGA*A) and *EagI* (5' C*GGCCG; 3' GCCGG*C) respectively. Using a methylation sensitive enzyme allows us to distinguish between methylated and unmethylated *FMR1* alleles. Normal samples will be showed clear band of 2,8 Kb relating to unmethylated normal allele and 5,2 Kb reflecting methylated normal allele due to X-inactivation process respectively. Premutation allele will be shown higher band above 2,8 Kb in permutation range. Full mutation allele will be shown an expanded allele above 5,2 Kb. Premutation allele and full mutation allele are unstable, therefore these allele sometimes might be identified as a smear. The fragmented DNA was electrophoresed in agarose gel. Afterward fragmented DNA in the agarose gel was transferred into nylon membrane. Then DNA was denatured with alkali and fixed into membrane using UV-crosslink apparatus. Subsequently single-stranded DNA was hybridized with probe pAO365 (tc **gag cgc ccg cag ccc acc**) and specific labeled radioactive (^{32}P)⁴³. Sequences of the probe in the FMR-1 gene are described below this section. Then X-ray film was used to develop the signal from hybridized DNA⁴¹.

TACACCCGCA GCGGGCCGGG GGTTCGGCCT CAGTCAGGCG CTCAGCTCCG 146801150

Primer Forward

TTTCGGTTTT ACTTCCGGTG GAGGGCCGCC TCTGAGCGGG CGGCGGGCCG 146801200

ACGGCGAGCG CGGGCGGCGG CGGTGACGGA GGCGCCGCTG CCAGGGGGCG 146801250

TGCGGCAGCG *CGGCGGCGGC GGGCGGCGGC GCGGCGGCGG AGGCGGCGGC* 146801300

GGCGGCGGCG GCGGCGGCGG CTGGGCCTCG AGCGCCCGCA GGCCACCTCT 146801350

Probe Sequence

CGGGGGCGGG CTCCCGGCGC TAGCAGGGCT GAAGAGAAGA TGGAGGAGCT 146801400

Primer

GGTGGTGGAA GTGCGGGGCT CCAATGGCGC TTTCTACAAG GTACTTGGCT 146801450

Reverse

Figure 1. Sequence of *FMRI* gene

Sequence of *FMRI* gene showed the location of both primers (Bold and underlined) and sequence of pAO365 probe (Underlined) and the CGG repeats sequence (bold and italic)

Procedures:

DNA Digestion

Seven ug DNA (in 35 uL diluted solution with milliQ) was digested with restriction endonuclease enzyme (1,25 uL Hind III and 1,5 uL Eag I) in the buffer React H together with 20 mM Spermidine in a total volume of 15,75 uL for a minimum 5 hours at 37°C.

Electrophoresis

Five uL loading buffer (bromophenol blue) was added to Digested DNA and subsequently 50 uL was loaded per well. Thirty uL of 0,5% ethidium bromide was added to the buffer.

Electrophoresis of DNA was performed 16 hours (overnight) in a 0,8% agarose gel at 38 volt in TAE buffer.

DNA transfer

DNA in agarose gel was transferred into nylon membrane (Genescreen Plus Perkin Elmer) using a 0,4N NaOH+NaCl buffer solution for capillary blotting using filter membrane, paper stack and weighing mass (~ 500 grams) above the membrane for five hours. Then, the nylon membrane was washed in the phosphate buffer, dried and cross-linked into a 1200 UV Stratalinker.

Radioactive labeling

Labelling beads were prepared by adding a tube Ready-To-Go DNA labelling beads (-dCTP) (Amersham) to 20 uL of water and incubating this solution for 5-60 minutes on ice. In the meanwhile the probe mix (pAO365) was denaturated at 95°C for 5 minutes. Thereafter the Probe-mix was mixed with the labelling beads and 2,5 uL (alpha-32-P-dCTP) was added for incubation overnight. A sephadex G-50 column was used to purify the labelled probe. Oligonucleotide labelling products were denaturated at 95 °C for 5 minutes. The oligonucleotide labelling products were added to a hybridization buffer. Hybridization was performed overnight at 65 °C. Tough cleaning was performed in 0,1 % SDS + phosphate buffer twice at 65°C for 5 minutes and once at 65 °C for 20 minutes. And

finally only phosphate buffer was used to end the washing procedures.

Autoradiogram

Autoradiography was performed on X-ray film (Kodak ECL) for 1-3 days at -80 °C using intensifications screens.

III.3.2.4. Multiplex Ligation-Dependent Probe

Amplification (MLPA)

Brief method description:

MLPA is a new method for relative quantification of about 40 different DNA sequences in an easy to perform reaction. This technique has been developed and first described by MRC-Holland⁴⁰. The different steps of MLPA are: DNA denaturation, hybridization of the DNA sample with a specific probe, ligation of the hybridized probe with a thermostable ligation and finally a PCR reaction is used to amplify the probe.

III.3.2.4.1 Sub-telomeric duplication and deletion (STD)

analysis using MLPA technique (P070 probe-kit)

Brief method description:

To detect STDs in samples, P070 probe-kit was used. P070 probe-kit contains one probe for each subtelomeric region from chromosome 1-22 and the two X/Y pseudo autosomal regions (PAR). For acrocentric chromosomes (13, 14, 15, 21 and 22) the probe for p-arm are located in the q-arm close to the centromere (Figure.2)

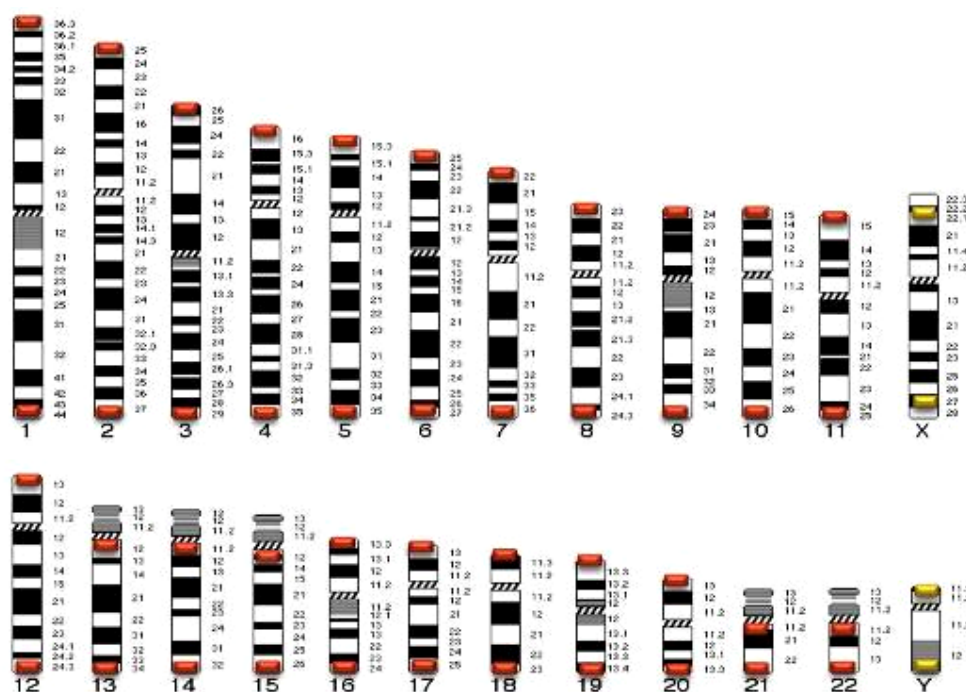


Figure 2. Probe locations in each chromosome.

Red block represent probe in autosome and yellow block represent probe in sex chromosome (probe in the Pseudo Autosomal Region/ PAR)

Procedures:

Denaturation

Approximately 200 ng DNA samples in 5 uL were denatured at 98 °C for 5 minutes followed by cooling to 25 °C.

Hybridization

One and half micro liter probe-mix mixtures were added to the denatured DNA at 25 °C and heated for 1 minute at 95 °C followed by incubation at 60 °C overnight. The probe-mix consisted of : 1,5 uL SALSA probemix (probe number P070) and probemix buffer (MRC-Holland).

Ligation

After overnight incubation, the temperature was reduced to 54 °C and in the meanwhile ligase-mix consisting of 3 µl Ligase-65 buffer A (transparent cap), 3 µl Ligase-65 buffer B (white cap), 25 µl milliQ and 1 µl Ligase-65 (brown cap) was added to the hybridized probes. This was followed by incubation of 15 minutes at 54 °C and after that heated for 5 minutes at 98 °C.

Amplification

Five uL ligated DNA was amplified in a final volume of 25 uL PCR solution containing 2 uL PCR buffer, 1 uL enzyme buffer, 1 uL PCR primers, 0,25 uL Taq Polymerase and 15,75 uL milliQ. The PCR conditions were as follows: initial denaturation at 97 °C for 1 minute followed by 35 cycles: 30 seconds 95 °C; 30 seconds 60 °C; 60 seconds 72 °C and ended with 20 minutes incubation at 72 °C.

Analysis

PCR products were mixed with LIZ 500 size standard marker and formamide and sent to the ABI 3730 machine to perform probe quantification. One uL PCR product was mixed in a final volume of 10 uL mix solution containing 8,7 uL formamide and 0,3 uL LIZ 500 standard size marker¹². Raw data from ABI 3730XL were analyzed using Genemapper Software version 4.0 (Apache Software). Subsequently the results from genemapper were imported and analyzed using a specific template for each probe developed in Microsoft Excel 2007.

III.3.2.4.2 Prader-Willi/Angelman Syndrome analysis using methylated specific MLPA technique (P028 Probe-kit)

Brief method description

To detect PW/AS in the samples P028 methylthion specific probe-kit was used. This probe-kit contains probes in the PW/AS region. In contrast with other MLPA methods, in the ligation procedure each sample was separated into two new tubes. In the new tube one tube was added with restriction enzyme (*Hha-I*) and another tube without restriction enzyme.

Procedures:

Denaturation

Approximately 200 ng DNA samples in 5 uL were denatured at 98 °C for 5 minutes and followed by cooling until 25 °C.

Hybridization

One and half micro liter probe-mix mixtures were added to the denatured DNA at 25 °C and heated for 1 minutes at 95 °C followed by incubation at 60 °C overnight. The probe-mix consisted of: 1,5 uL SALSA probemix (probe number me028) and probemix buffer (MRC-Holland).

Ligation, splitting and digestion with restriction enzyme

After overnight incubation, the temperature was reduced to 49 °C and meanwhile hybridized probe were added with ligase-mix that consisting of the 3 µl Ligase-65 (MRC-Holland)

buffer A (transparent cap) and 10 µl milliQ then solution were divided into two different tubes each tube consisting 10 µL solution. For first tube were added with 1,5 µl Ligase-65 buffer B (white cap), 8,25 µl water and 0,25 µl Ligase-65 (brown cap) whilst second tube were added with 1,5 µl Ligase-65 buffer B (white cap), 7,75 µl milliQ, 0,25 µl Ligase-65 (brown cap) and 0,5 µL *Hha-I* restriction enzyme (Methylated) this was followed with incubation for 30 minutes at 49 °C, and subsequently heated for 5 minutes at 98 °C.

Amplification

Five µL ligated DNA was amplified in final volume of 25 µL PCR solution containing 2 µL PCR buffer, 1 µL enzyme buffer, 1 µL PCR primers, 0,25 µL Taq Polymerase and 15,75 µL milliQ. The PCR conditions were as follows: initial denaturation at 72 °C for 1 minutes followed by 35 cycles: 30 seconds 95 °C; 30 seconds 60 °C; 60 seconds 72 °C and ended with 20 minutes incubation at 72 °C.

Analysis

PCR products were mixed with LIZ 500 size standard markers and formamide and sent to the ABI 3730 machine to perform probe quantification. One µL PCR product was mixed in a final volume of 10 µL mix solution containing 8,7 µL formamide and 0,3 µL LIZ 500 standard size. Subsequently the results

from genemapper were imported and analyzed using a specific template for each probe developed in Microsoft Excel 2007.

III.3.2.5 DNA purification

Some DNA samples that could not be analyzed by MLPA technique need to be purified. Purification was carried out using a QIA cube apparatus. Approximately 10 ng DNA was diluted in total 200 uL PBS. To each sample 4 uL of RNA-ase was added. Subsequently the diluted DNA was purified in a QIA cube apparatus as recommended by QIA cube manufacture.

III.3.2.6 Sequencing

A false positive result might be caused by DNA change (polymorphisms or mutations) in the patient's DNA at the location of the probe (So hybridization cannot take place). Therefore to exclude this false positive, sequencing of DNA surrounding the probe was performed. One uL DNA (~100 ng) of each sample was diluted in a final volume of 15 uL PCR mix solution, containing 7,5 uL Fastmix PCR Solution (Applied Biosystem), primers for each probe (P070 specific location sequence probe) and 6 uL water. The mixture was amplified in a Veriti Thermocycler (Applied Biosystem) under the scheme of fast PCR by initial denaturation at 95 °C for one minute followed by 35 cycles of 94 °C, 10 sec; 62 °C, 25 sec and ended with incubation at 72 °C for 1 minute. The sequencing products were purified using Millipore columns as

recommended by the manufacturer. The products were dried and diluted with 20 uL TE buffer. One and half uL purified DNA was mixed with M13 primer forward and reverse. Finally, M15 Big terminator Dye (Applied Biosystem) added to the diluted sequencing products and analysed on an ABI 3730 XL sequencer (Applied Biosystem).

III.3.3 Confirmation with another test

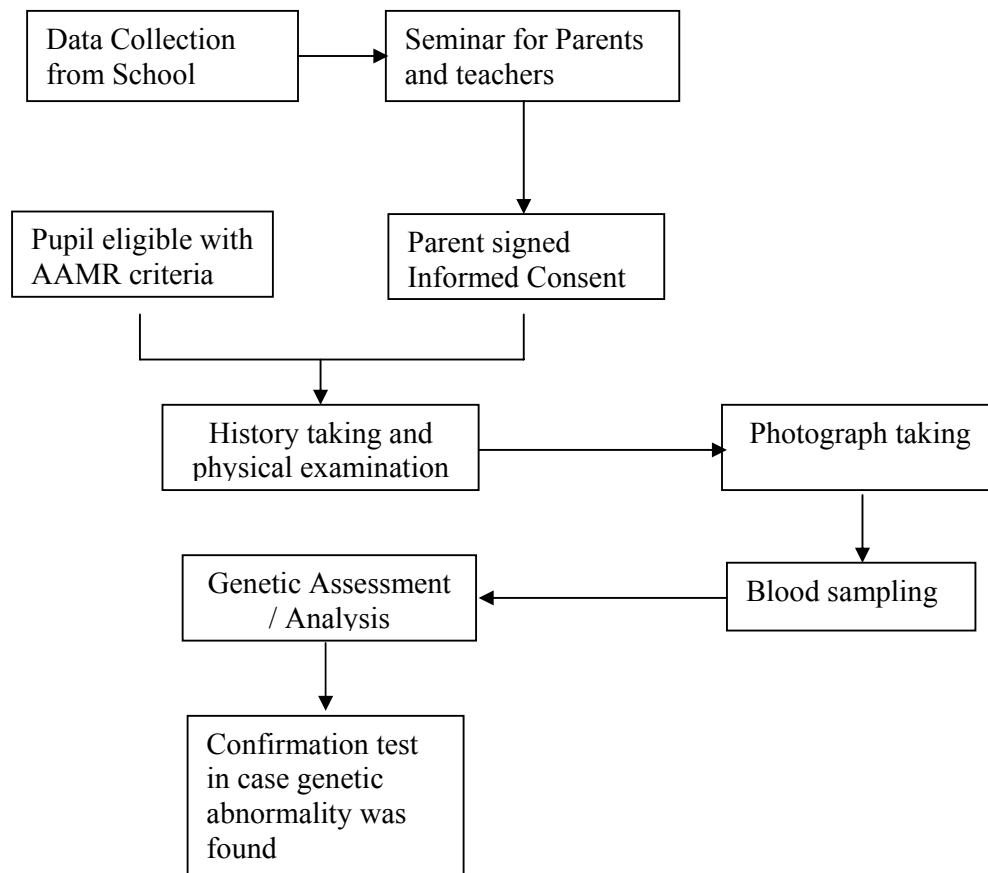
Some results need to be confirmed with another probe-kit. A positive result of STD analysis using probe P070 were repeated and confirmed using probe P036D in The DNA Diagnostic Department RUNMC, The Netherlands. Gene locations in each probe are described in Table 3. One sample needs to be confirmed using another probe P096 (syndromal kits). Reanalysis with P070 and P036D probe-kit and radio-labeling procedures in Southern Blotting Analysis were performed by experienced technicians (GS and MRV) following a standard operational protocol from the Department of DNA Diagnostics of the RUNMC.

Table 3. Gene location differences in Probe P070 and P036D

P070		P036D	
Chromosome	Gene location	Chromosome	Gene location
1q	KIAA1720	1q	KIAA1720
2q	APG4B	2q	CAPN10
3q	KIAA0226	3q	BDH
4q	FRG1	4q	FRG1
5q	GNB2L1	5q	GNB2L1
6q	TBP	6q	PSMB1
7q	VIPR2	7q	VIPR2
8q	RECQL4	8q	KIAA0150
9q	EHMT1	9q	EHMT1
10q	ECHS1	10q	PAO
11q	KIAA1030	11q	KIAA0056
12q	ZNF10	12q	ZNF10
13q	CDC16	13q	F7
14q	MTA1	14q	MTA1
15q	FLJ22604	15q	ALDH1A3
16q	GAS11	16q	GAS11 / GAS8
17q	SECTM1	17q	TBCD
18q	CTDP1	18q	FLJ21172
19q	BC-2	19q	BC-2
20q	FLJ20517	20q	OPRL1
21q	S100B	21q	HMT1
22q	ARSA	22q	RABL2B
Xq (PAR2)	SYBL1	X/Yq	SYBL1
1p	TNFRSF18	1p	SCNN1D
2p	ACP1	2p	ACP1
3p	CHL1	3p	CHL1
4p	ZNF141	4p	FLJ20265
5p	LOC133957	5p	PDCD6
6p	IRF4	6p	IRF4
7p	UNC84A	7p	CENTA1
8p	FBXO25	8p	FBXO25
9p	FLJ00026	9p	DMRT1
10p	BS69	10p	KIAA0934
11p	BET1L	11p	RIC-8
12p	RBBP2	12p	SLC6A12
“13p”	PSPC1	“13p”	PSPC1
“14p”	ADPRTL2	“14p”	HEI10
“15p”	NDN	“15p”	MKRN3
16p	DEC2	16p	POLR3K
17p	RPH3AL	17p	RPH3AL
18p	THOC1	18p	USP14
19p	PPAP2C	19p	CDC34
20p	FLJ22115	20p	SOX12
“21p”	STCH	“21p”	RBM11
“22p”	IL17R	“22p”	BID
Xp (PAR1)	SHOX	X/Yp	SHOX

For acrocentric chromosomes (13, 14, 15, 21 and 22) the probe for p-arm are located in the q-arm close to the centromere Gene location for each probe available in MRC-Holland website (www.mrc-holland.com)

III.4 Research Scheme



III. 5. Variables

- Clinical sign of Mental Retardation (Dysmorphology)

Scale : Nominal

- Cytogenetics and molecular assessment result

Scale : Nominal

III.6. Operational definitions:

1. Clinical signs (phenotype / dysmorphology) : All clinical features that occur together with the MR
2. Genetic assessment result: all chromosomal rearrangements or gene mutations that cause MR

3. Mental Retardation criteria (AAMR)⁸:

- i. Substantial limitations in present functioning (subaverage intellectual functioning i.e IQ < 70)
- ii. Limitations in two or more of the following applicable adaptive skill areas: communication, self-care, home living, social skills, community use, self-direction, health and safety, functional academics, leisure and work.
- iii. The age of onset should be before age 18⁸

4. Level of Mental retardation⁸:

Class	IQ
Profound mental retardation	Below 20
Severe mental retardation	20–34
Moderate mental retardation	35–49
Mild mental retardation	50–69
Borderline mental retardation	70–79

III.7 Collected Data

III.7.1 Primary Data:

1. Personal identity of each mentally retarded subject, including date of birth, sex, ante-natal care, pre-natal care and family tree (pedigree).
2. Data on clinical signs of the individuals. These data were obtained according a diagnosis protocol check list.

III.7.2 Secondary data:

Medical records which were obtained before the pupils entered special school (IQ etc).

III. 8. Data Analysis:

Data will be analyzed with descriptive method then data will be presented in tables and graphics.

CHAPTER IV

RESULTS

From 186 pupils selected in three special schools, only 122 pupils can be included in further analysis based on the inclusion and exclusion criteria. A total of 122 samples were obtained from subjects with mental retardation from three special schools in Semarang as indicated in Table 4. Cytogenetic preparations were carried out in all samples. All samples were screened for CGG repeats in the *FMR-1* gene. Eleven samples (9 females with single alleles and 2 males; 1 with absence or a PCR product and 1 with high CGG repeat) had to be confirmed by southern blot.

Of the 122 samples, 121 were suitable for sub-telomeric deletion and duplication studies (STDs) with MLPA analysis, whereas 1 sample was excluded based on the *FMR-1* analysis results. However, one sample cannot be analyzed using MLPA analysis due to bad DNA

Of these 107 samples (1 sample was excluded based on FMR-1 result, 1 sample due to Down Syndrome and 13 based on STDs analysis), 13 were selected for Prader-Willi/Angelman syndrome (PW/AS) study. This selection was based on the phenotypic features of the patients.

IV.1. *FMR-1* Analysis

A total of 122 subjects with developmental disability from three special schools in Semarang were analyzed for trinucleotides repeats expansion (PCR

Analysis on agarose and genescan). To determine exact number of CGG repeats, PCR products were analyzed by Fragment Length Analysis (genescan). Southern Blotting was used when the PCR / Fragment Length Analysis were not clear; there was no PCR product in agarose gel (1 sample), or when the CGG repeats were too high (1 sample) and when single allele (can be homozygous samples or normal and expanded allele) found in woman samples (9 Samples). Southern Blot analysis was performed in those samples which identified one subject with a full CGG expansion, one subject with a mosaic pattern (premutation and full mutation) and none of the females were found to have a CGG expansion. The *FMR-1* CGG repeat sizes of screened subjects are depicted in Table 5 while the results of Southern Blotting are illustrated in Table 6. Furthermore the graphic of the repeat sizes in screened subject by PCR analysis for *FMR-1* (genescan result) is depicted in Figure 3.

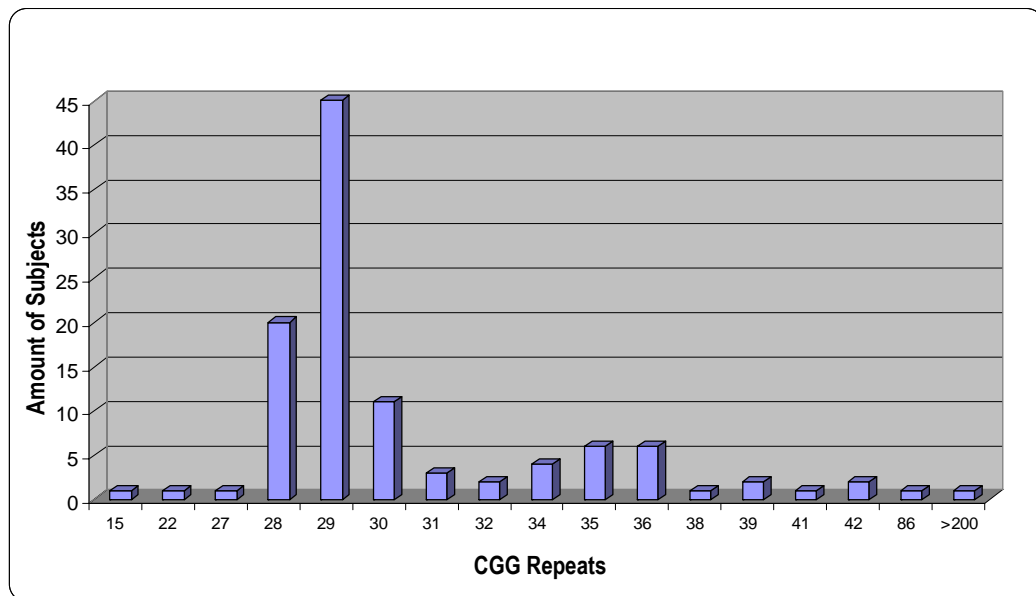
Table 5. CGG repeat sizes in subjects screened by *FMR-1* Analysis

CGG Repeat	Male	Female *)
15-35	80	24
36-55	12	4
56-200	1	-
> 200	1	-

*) In female samples only the highest repeat allele was counted.

Table 6. Results of molecular analysis

No.	Pupil ID	Sex	PCR result	Southern Blot Result
1.	SM-30C	M	No product	> 5,2 Kb
2.	SM-34C	M	High CGG Repeat	> 2,8 Kb ~ > 5,2 Kb
3.	SM-20	F	Single allele	2,8 Kb & 5,2 Kb
4.	SM-37C	F	Single allele	2,8 Kb & 5,2 Kb
5.	SM-26	F	Single allele	2,8 Kb & 5,2 Kb
6.	SM-36C	F	Single allele	2,8 Kb & 5,2 Kb
7.	N-52	F	Single allele	2,8 Kb & 5,2 Kb
8.	N-54	F	Single allele	2,8 Kb & 5,2 Kb
9.	N-61	F	Single allele	2,8 Kb & 5,2 Kb
10.	PI-19	F	Single allele	2,8 Kb & 5,2 Kb
11.	PI-21	F	Single allele	2,8 Kb & 5,2 Kb

Figure 3. Distribution of CGG repeats among all subjects.

The most frequent CGG repeat in this population was 29 CGG repeat, then followed by 28,30,35 & 36 respectively.

This study showed, an allele with 29 CGG repeats is the most frequent one in this population followed by 28,30,35 & 36 CGG repeats as illustrated in Figure 2.

IV.2.STDs Analysis with MLPA technique

To identify sub-telomeric deletions and duplications (STDs) analysis by MLPA was carried out. One male was excluded for STDs analysis because of a positive FMR-1 result. However one sample cannot be analyzed using MLPA analysis due to bad DNA. Therefore 120 samples were tested with MLPA analysis. The initial screening with the P070 kit (MRCH) which contains probes in all telomeric regions of the different chromosomes showed 13 samples with subtelomeric rearrangements as indicated in Table 7. Re-analysis using another probe-set (P036D) by DNA Diagnostic RUNMC confirmed that 8 out of 13 indeed had a sub-telomeric rearrangement. One sample was excluded from further analysis after the result of MLPA was confirmed with cytogenetics showing trisomy 21 (Down syndrome). Three samples that did not showed same aberration by confirmation with P036D probe were considered most likely because of polymorphism but it still could be something (very small deletion / duplication) and it will be discussed in the next chapter. The results of STDs analysis using MLPA technique are shown in Table 7.

Table 7 Sub-telomeric deletions and duplications found in subjects by STDs**MLPA Analysis using P070 kit**

No.	Subject ID	Sex	Aberration(s)	P036D performed?	Result
1	N-10 / ID 153	M	Del 4pter / dup 8pter	Yes ¹⁾	Confirmed
2.	PI-14 / ID 207	M	Dup 2pter	Yes	Confirmed
3	PI-22 / ID 398	F	Del 18pter	Yes	Confirmed
4	N-26 / ID 169	M	Dup 16qter	Yes	Confirmed
5	N-55 / ID 386	F	Del 10pter / dup 9pter	Yes	Confirmed
6	SM-36C / ID 419	F	Del 18 pter/ dup 4pter	Yes	Confirmed
7	N-47 / ID 190	M	Dup 9pter	Yes	Confirmed
8	P1-24 / ID 400	M	Del XYqter	No (P070 twice)	P036D not performed
9	N-38 / ID181	M	del4qter/dup10qter	Yes ²⁾	Not confirmed
10	N-18 / ID 161	M	del4qter/dup10qter	Yes ²⁾	Not confirmed
11.	N-34 / ID 177	M	Dup 21 pter/qter	No (Cytogenetic)	Confirmed ⁴⁾
12	N-39 / ID 182	M	Dup 10pter	Yes ³⁾	Not confirmed
13	N-30 / ID 173	M	Dup 10qter	Yes ³⁾	Not confirmed

¹⁾ Confirmed with P096 kit (syndromatic kit) and sequencing also

²⁾ Samples analyzed with P036D first and confirmed with P070
(The other way around)

³⁾ Artificial result cannot be excluded

⁴⁾ Trisomy 21

IV.3. PW/AS Analysis with MLPA Technique

Thirteen samples from a total of 106 subjects were selected to be analyzed for PW/AS syndrome using the P028 kit (MRCH). Selection was based on clinical examination criteria for PWS/AS. No positive result was detected in the samples both in the methylated and unmethylated probes.

IV.4. Clinical findings

Before the molecular analyses were carried out, clinical examination had been performed in all subjects. These were performed using a standardized protocol adapted from the RUNMC. Height, length and OFC were measured and dysmorphisms were described. In male subjects testicular size was measured using a comparative palpation method with the Prader orchidometer. The phenotypic features of all subjects with a proven genetic abnormality are presented in Table 8 and the clinical photographs of various subjects with sub-telomeric rearrangements are presented in Figure 4.

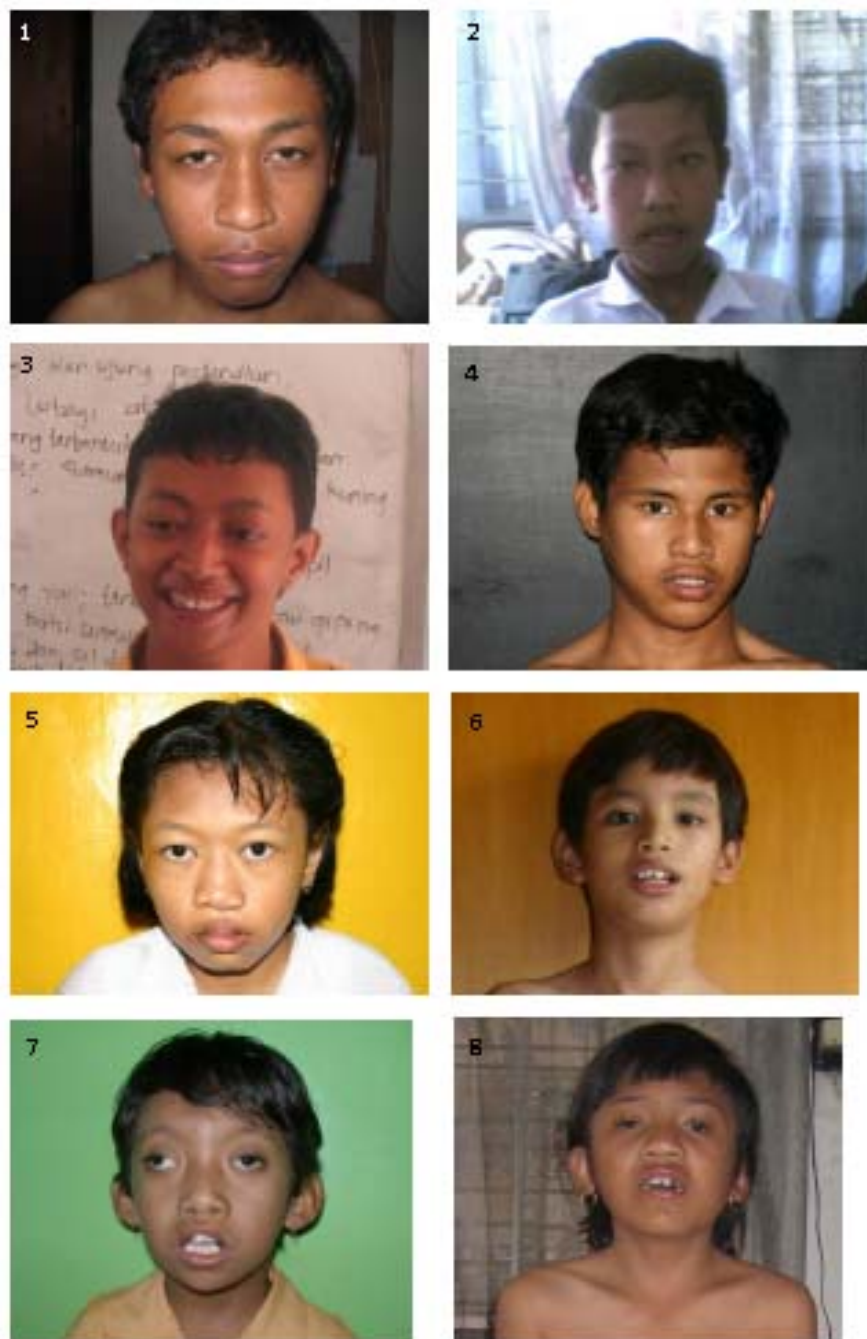


Figure 4a. Clinical photographs of subjects (number 1-8)
 Numbers in the photographs correspondent to the clinical features,
 cytogenetics results and molecular result as described in the Table 8.

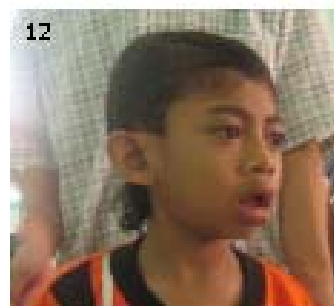
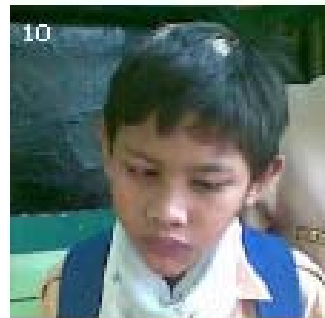


Figure 4b. Clinical photographs of subjects (number 9 – 15)
Numbers in the photographs correspondent to the clinical features,
cytogenetics results and molecular result as described in the Table 8.

Table 8. Summary of Dysmorphological features

	Patient 1 SM-30C	Patient 2 SM-34C	Patient 3 N-10 / ID 153	Patient 4 PI-14 / ID 207	Patient 5 PI-22 / ID 398
Laboratory Result	FMR-1 Full mutation	FMR-1 Mosaic (PM and FM)	Del 4pter / Dup 8 pter	Dup 2pter	Del 18pter
Gender	M	M	M	M	F
Age	19	15	19	17	12
Mental retardation	Mild	Mild	Moderate	Moderate	Mild
Hypotonia	No	No	Yes	No	No
Height	P3-P98	P3-P98	< P3	P3-P98	P3-P98
OFC	P3-P98	P3-P98	< P3	P3-P98	< P3
Behaviour problems	Shyness	Shyness			
Other features					
Cranial/Face	Long and narrow face	Long and narrow face	greek helmet	Brachycephaly	
Ears		Big and prominent	low set	Posteriorly rotated	Prominent anti-helix
Ocular region			strabismus telecanthus	Upslanting palpebral fissures Deep set eyes Strabismus	hypertelorism
Nose			Broad base, pointed		Flat nose Broad Nasal bridge Deviated columnella
Mouth area			Wide Mouth Thick lips		Cleft lip (operated) Full lips
Extremities	Long finger				
Thorax				Pectus Excavatum	
Heart					
Genital	Macroorchidism				
Skin					
Other features				Delayed speech Poor coordination	

Table 8. Summary of Dysmorphological features (Continued)

	Patient 6 N-26 / ID 169	Patient 7 N-55 / ID 386	Patient 8 SM-36C / ID 419	Patient 9 N-47 / ID 190	Patient 10 P1-24 / ID 400
Laboratory Result	Dup 16q	Del 10pter / dup 9pter	Del 18 pter/ dup 4pter	Dup 9pter	Del XYqter
Gender	M	F	F	M	M
Age	11	7	12	20	10
Mental retardation	Mild	Moderate	Moderate	Mild	Moderate
Hypotonia	No	No	No	No	No
Height	P3-P98	< P3	< P3	P3-P98	P3-P98
OFC	P3-P98	< P3	< P3	P3-P98	P3-P98
Behaviour problems	Autistic Aggressive				Aggressive
Other features					
Cranial/Face	High forehead	Brachycephaly		Prominent forehead Retrognathia	Long and narrow
Ears		Low-set, Prominent	posteriorly rotated		
Ocular region	Telecanthus Thick eyebrows	Ptosis Strabismus Telecanthus	ptosis telecanthus strabismus downslanting palpebral fissure	Deep-set eyes telecanthus	Strabismus telecanthus
Nose		Broad base	Depressed nasalbridge		Broad base
Mouth area	Short philtrum Teeth overbite High arched palate	Short philtrum Cleft lip	Down turned of mouth	Short philtrum	Wide mouth Mouth hypotonia Thick lips
Extremities	Tapering fingers Clinodactily 5th finger	Sandal gaps	Cubitus valgus Short 5th finger	Single palmar crease	Single palmar crease
Thorax			Wide-spaced nipples		
Heart					
Genital					
Skin					
Other features					

Table 8. Summary of Dysmorphological features (Continued)

	Patient 11 N-38 / ID181	Patient 12 N-18 / ID 161	Patient 13 N-34 / ID 177	Patient 14 N-39 / ID 182	Patient 15 N-30 / ID 173
Laboratory Result	del4qter/dup10qter	del4qter/dup10qter	Dup 21 pter/qter	Dup 10pter	Dup 10qter
Gender	M	M	M	M	M
Age	11	9	9	10	13
Mental retardation	Mild	Mild	Moderate	Mild	Mild
Hypotonia	No	No	No	No	No
Height	P3-P98	P3-P98	P3-P98	P3-P98	P3-P98
OFC	P3-P98	P3-P98	P3-P98	P3-P98	P3-P98
Behaviour problems		Autistic	Autistic		
Other features					
Cranial/Face			Alopecia areata	Brachycephaly	
Ears	Low-set (slight)		Low-set	Posteriorly rotated, Large	
Ocular region	telecanthus	Epicantal fold	Hypertelorisme Small palpebral fissure Nystagmus	Telecanthus	Telecanthus Upslanting palpebral fissure Strabismus
Nose			Flat nasal bridge		Depresed nasal bridge
Mouth area				Full lips Down-turned	
Extremities			Simian crease Short 5th finger Sandal gap		
Thorax					
Heart					
Genital					
Skin					
Other features					

IV.5 *FMR-1* analysis of a family with a full mutation.

The family of the proband with a full CGG expansion was visited and blood samples were taken from all family members in order to detect carriers and possibly affected individuals. *FMR-1* gene analysis including Fragment Length Analysis and Southern Blot were performed in all family members as shown in the pedigree in Figure 5. The *FMR-1* analysis results are illustrated in Table 9.

Figure 5. Pedigree of affected Fragile-X family (SM-30C)

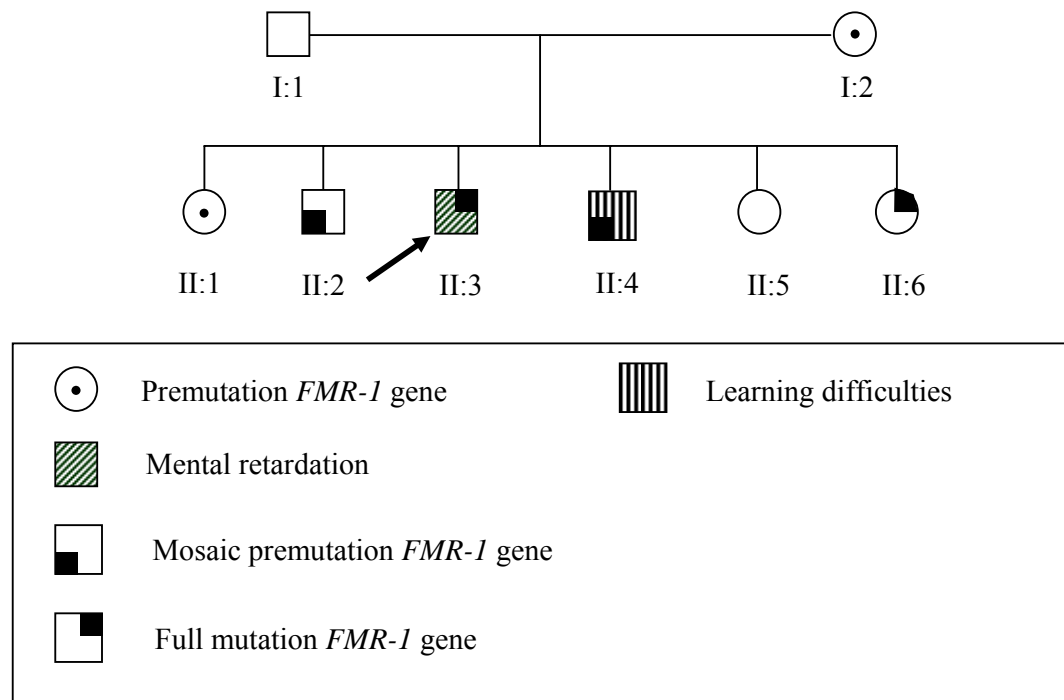


Table 9. *FMR-1* test in family members from subject with full CGG repeat expansion (subject II:3/SM-30C)

Subject ID	Pedigree code	Sex	CGG Repeat	Remarks
ID 572	I:1	M	26	Normal
ID 573	I:2	F	29/69	Premutation carrier
ID 574	II:1	F	26/86	Premutation carrier
ID 575	II:2	M	86-103	Mosaic permutation carrier
ID 413	II:3	M	>200	Full mutation (Tested before / SM-30C)
ID 576	II:4	M	80-103	Mosaic permutation carrier
ID 577	II:5	F	26/ 29	Normal alleles
ID 578	II:6	F	26/>200	Full mutation

Several carrier females were identified in this family; the mother (I:2) had a normal allele and an elevated band size in PCR amplification which was confirmed by Fragment Length Analysis and Southern Blot. The first daughter (II:1) had 1 normal allele and 1 allele in the premutation range. Two brothers (II:2 and II:4) of the proband had a mosaic premutation allele which was assessed by PCR and fragment length analysis and confirmed by Southern Blot. The second daughter (II:5) had normal alleles. The youngest daughter (II:6) showed 1 normal allele and 1 fully expanded allele which was assessed by Southern Blot. Except for the affected proband all sibs and the carrier mother apparently had a normal

intelligence. However, one male (II:4) had learning difficulties which will be discussed in the next chapter.

IV.6 Cytogenetic Analysis

Cytogenetic preparations were carried out in all samples; One sample with trisomy 21 was detected, two with structural abnormalities and the others showed normal karyotype. The complete result of cytogenetics analysis showed in the appendix section.

Table 10. Results of cytogenetics analysis

No.	Subject ID	Sex	Karyotype
1.	N-34	M	47,XY+21
2.	N-10	M	46,XY,del(4)(p16)
3.	PI-14	M	46,XY,add (2)(p25)

CHAPTER V

DISCUSSION

The purpose of this study was to identify the major genetic causes of mental retardation in Indonesia and to establish its prevalence. The study was conducted in 1.5 year and the subjects were tested for microscopic visible chromosomal aberrations, sub-telomeric deletions and duplications, Fragile X syndrome, Prader-Willi and Angelman syndrome. In addition, the results of this research project should raise the level of awareness in the Indonesian community, especially within the medical society particularly among clinicians.

Many factors, such as economic factors, parental education and a low awareness, may lead to a failure in registering mentally retarded children at special schools. This may have led to an ascertainment bias in selecting the individuals for this study because the total number of subjects that were registered at special schools might not have represented the total number of persons with mental retardation in Indonesia.

The molecular identification of most major causes of mental retardation had to be performed abroad because of the lack of laboratory facilities in Indonesia. To resolve this issue, this part of the project was done in the RUNMC in the Netherlands.

Finally, it has to be taken into account that, due to financial and time constraint, the progress of this research was not as smooth as it was planned to be. Sometimes the different steps of the research project were performed within

inappropriate order. For instance, one trisomy 21 subject was identified by MLPA before it was confirmed by regular cytogenetic analysis.

V. 1. Fragile-X Syndrome (FXS)

The finding of a sample with a CGG expansion confirmed that FXS is one of the causes of mental retardation in the Indonesian population. Based on the results of this research, the prevalence of FXS in this population is 0,82 % (1/121) and 1,07% (1/93) among the male part of the Indonesian inhabitants respectively (Down syndrome case was excluded from calculation). Possibly, the prevalence was higher because there was another subject with a mosaic pattern of a pre-mutation and a full mutation. Rousseau et al. (1994) observed no significant mental status difference between subjects with a mosaic mutation pattern and a full mutation⁴⁵. Based on that study this subject with mosaicism should also be included in counting the prevalence of FXS. If so, the prevalence would be 1,65% (2/121) in the total Indonesian population and 2,15% (2/93) in the male population. Previous research by Faradz et al. (1999) showed a lower FXS prevalence of 1.9 % (5/262) in the male population⁵. This lower prevalence might have been produced because a larger number of samples were used in Faradz's study.

V.1.1. Clinical findings of subject with CGG expansion

One subject with a full expansion of the CGG repeat had major clinical sign of FXS i.e. mental retardation, shyness behavior, a long face, a pointed chin,

long fingers and macroorchidism. However he did not have macrocephaly, loose joints, and a soft stretchy skin which are major physical features of FXS ⁴⁶. In addition, he also did not have large and prominent ears which is one of the classic triad of Fragile-X Syndrome.

One subject with a mosaic pattern of a premutation to a full mutation was identified. During clinical assessment he was noted with shy behavior, a long and narrow face and large and prominent ears. He had two out of three classic features of FXS. Regardless of the fact that mosaic FXS males still produce FMR protein, they are nevertheless developmentally delayed ⁴⁵.

One explanation could be that the amount of FMR-1 protein present in the brain is insufficient to permit normal development. Furthermore in the Southern Blot analysis of this subject was showed that full mutation allele was more dominant than premutation allele. Nevertheless Cohen et al. (1996) observed that mosaic males had a 2-4 times higher rate of adaptive skills than non-mosaic Fragile-X males⁴⁷. Unfortunately we did not test the adaptive skills of our subjects yet.

V.1.2. Fragile-X in a family

Premutation alleles are not thought to be associated with clinical effects in the majority of the premutation carriers. However, some studies have suggested that a small proportion of the premutation carriers may have some behavioral, physical or cognitive features ^{48,49}. Furthermore premature ovarian failure (POF) was reported in about 20 % of the premutation carriers. Allingham-Hawkins et al Jacquemont et al, and Hagerman et al, also reported progressive intention tremors

and cerebellar ataxia in several male premutation carriers above the 50 years of age, called Fragile-X associated Tremor Ataxia Syndrome (FXTAS)⁵⁰⁻⁵³.

In addition, all premutation carriers are at risk to get FXS affected children. The chance that a female permutation carrier passes the X chromosome, with the fragile X permutation, over to each of her offspring is 50%. Since the trinucleotide repeats can expand upon transmission from a carrier female to her male offspring, her sons are at risk to inherit an expanded CGG mutation and subsequently to be affected by FXS⁴⁶.

The affected family described in this study is a perfect example to illustrate the X-linked inheritance of FXS. The mother had a premutation allele which was transmitted to almost all of her offspring, except for her second daughter who inherited her normal X chromosome. Interestingly, all offspring who inherited the affected allele had various repeat lengths varying from a premutation allele, a mosaic pattern within the range of premutation and a full mutation.

Two sons in this family also had a premutation mosaic pattern. All mosaic patterns were still in the premutation range in contrast with previous case with mosaic between premutation to full mutation. Thus the result has no consequences to their mental status. However, in fact one son has a premutation mosaic pattern developed learning disability. It is suggested that his learning disabilities was not due to his premutation allele. Furthermore to confirm this, FMRP studies is needed to evaluate the FMRP product.

It will be valuable to trace back all premutation carriers as far as possible in the previous generations to determine which persons in the family are at risk to produce affected offspring and to monitor the older premutation carriers for FXTAS (in male carriers) and POF (in female carriers). Family members should be aware of the X-linked inheritance pattern and they should be advised to take the test to determine carriership. In this case it was not possible to trace the premutation back to another generation because both parents of the mother who might have had a premutation were not available.

Genetic counseling should be provided to this family. The first daughter, with a premutation allele, is at risk to transmit the affected allele to the next generation. All daughters of the first son, who is also carrying a premutation, will be a premutation carrier. The youngest daughter is carrying a full mutation allele which means she may develop normally or will have learning disabilities or even some degree of mental retardation⁵⁴⁻⁵⁶

V.1.3 Distribution of CGG repeats in this population

This study showed that an allele with 29 CGG repeats is the most frequent one at FRAXA locus in this population. Numerous studies have reported an allele with 30 CGG repeats as the most frequent one at the FRAXA locus in Caucasian populations, and the 29 CGG repeats initially reported has been considered a miscalculation due to differences in C+G content which affect the migration of the PCR products^{57,58}. In the 119 non fragile-X subjects, we identified 15 different normal alleles ranging from 15 – 43 CGG repeats as showed in figure 2. In six

alleles (28,29,30,35,36) accounted for 75% of the total, and 29 CGG repeats is the most frequent one in our study, which is in contrast with Caucasian population (the most frequent is 30 CGG repeats)^{57,58}. In Asian population, 29 CGG repeats is reported to be the most frequent allele^{59,60}, although in another study suggested that 28 CGG repeats to be the most allele in China⁶¹. Nevertheless this study confirmed the similar result with previous study in Indonesian population by Faradz et al⁶.

V.2. STD's analyses

The first results of the Sub-telomeric deletion and duplication (STDs) studies in this population confirmed that STDs are a cause of mental retardation in the Indonesian population. Phenotypic manifestations of Sub-telomeric rearrangements vary and the clinical features depend on the size of the duplication and/or the deletion, on the chromosome number(s) affected and the genes located in this region. Koolen et al and Rooms et al. suggested that MLPA is a reliable method to detect sub-telomeric rearrangements. However, both studies considered the fact that confirmation with FISH is still necessary^{12,16}.

Confirmation studies are considered necessary to exclude a polymorphism under the probe. However, Nothrop et al. suggested that the strategy of using two sets of complementary probe sets would mostly overcome the polymorphism problems and this strategy was also conducted in this research project¹⁷. The P070 probe-kit was used in the first analysis and subsequently, to confirm possible positive results, the P036D probe-kit was used. In general, most of the

probe sequences of the P070-kit are located more distal to the telomeric region than the probes in the P036D-kit. Therefore, small submicroscopic deletions and duplications could still be identified. An example of the sub-telomeric probe positions of the two different kits is shown in Figure 6.

In addition, Nothrop et al. suggested that caution must be applied in distinguishing polymorphic from non-polymorphic copy number changes, because some deletions or duplications detected by one probe, which were not confirmed with another probe, could still be pathogenic¹⁷. For instance, if the identified deletion or duplication would be located in a known polymorphic region and only one probe would detect this abnormality, it might be considered as a polymorphism. However, if the aberration would be located in a non-polymorphic region it could still be a very small deletion or duplication. As explained before, the P070 probes are located more distally in the telomeric region. Therefore, in case a sample shows a positive result with the P070 probe, not detected in the P036D probe, it might still be a very small deletion in the most distal end of the telomere. However, this will not count for all chromosomes as not all P070 probes are located in the same gene or more distally in the telomere than the P036D probes. Thus, before a conclusion can be made, the location of every probe needs to be checked in the genome browser.

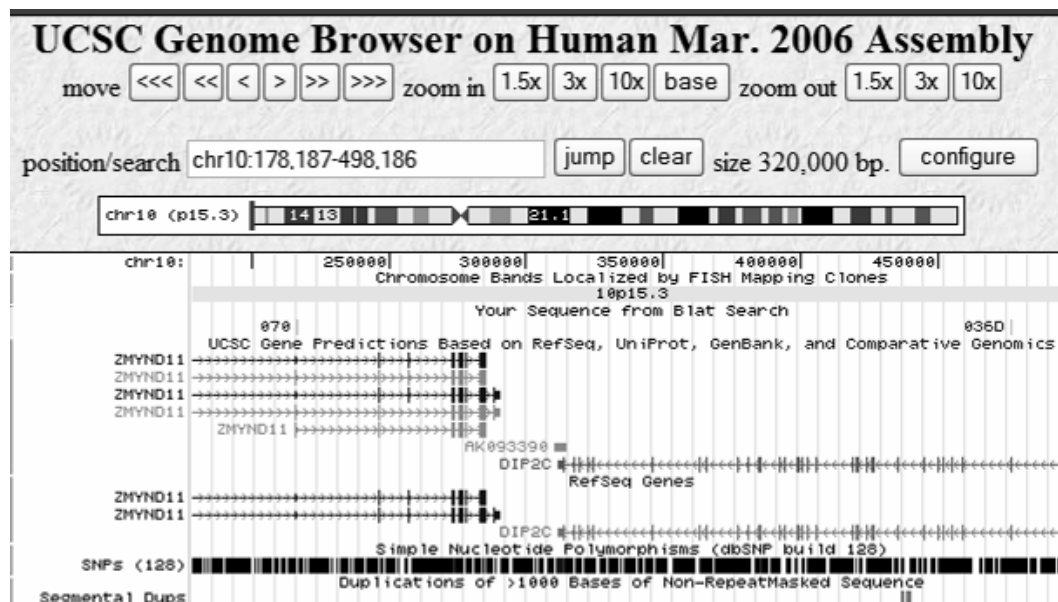


Figure 6. The probe location of P070 and P036D in the telomeric region of chromosome 10p (Adopted from the UCSC genome browser, Hg 19:<http://genome.ucsc.edu/>)

In addition, Koolen et al. suggested, to exclude polymorphisms, the interpretation of the MLPA results should always include parental testing and a comparison of the phenotype of the subject with the clinical features of previously reported subjects with similar sub-telomeric rearrangements¹². Due to technical reasons, parental testing could not be carried out in this study. However, it was possible to compare the clinical manifestations of some affected subjects with previously reported subjects with similar sub-telomeric rearrangements. In the following sections the clinical presentation of these subjects will be compared to the information available in literature and in chromosomal aberration databases.

V2.1 Subject 1 (Del 4pter/Dup 8pter)

Deletions of chromosome 4pter are well known and cause the characteristics of the Wolf-Hirschhorn Syndrome (WHS). The WHS Critical Region (WHSCR) is located on 4p16.3¹¹. Deletions with a size less than 3.5 Mb have been described by Zollino et al. and resulted in a distinct but relatively mild WHS⁶². In Subject 1 the P096 probe was used to investigate the size of the WHSCR deletion which showed a deletion of the whole WHSCR (Figure 7). WHS is associated with a characteristic face comprising wide mouth, short upper lip with flat philtrum, beaked nose, prominent eyes, telecanthus, slanted palpebral fissures and maxillary hypoplasia, MR, hypotonia, growth retardation and microcephaly^{62,63}. In addition to the 4pter deletion, 8pter duplication was also detected, which implies that this subject may have some characteristics of duplication 8pter. However, duplications of segment 8pter are associated with mild mental retardation only without typical dysmorphic features^{64,65}. He had growth retardation, microcephaly, hypotonia and some WHS facial characteristics. A clinical summary of this subject is shown in Table 8. Therefore we conclude that almost all clinical manifestations in this Subject are caused by a terminal deletion of chromosome 4p. The phenotypic features of this subject were suggestive of WHS which was confirmed by MLPA analysis using probe P070, P036D, and P096. Since a duplication of chromosome 8p does not lead to a distinct phenotype and because it is difficult to confirm either by MLPA or FISH it is suggested if this subject indeed has 8pter duplication also. Due to the presence both of deletion and duplication in this subject, unbalanced translocation is thought. Therefore cytogenetic result is needed to confirm microscopically

visible unbalanced translocation (to see whether it is present or not), however in the cytogenetic analysis only deletion of 4p16 was detected [This subject has karyotype of 46,XY,del(4)(p16)] . We suggest that duplication of 8pter is very small, thus FISH and or array is needed for further assessment. In addition, parental testing is needed to trace the balanced translocation in the parent.

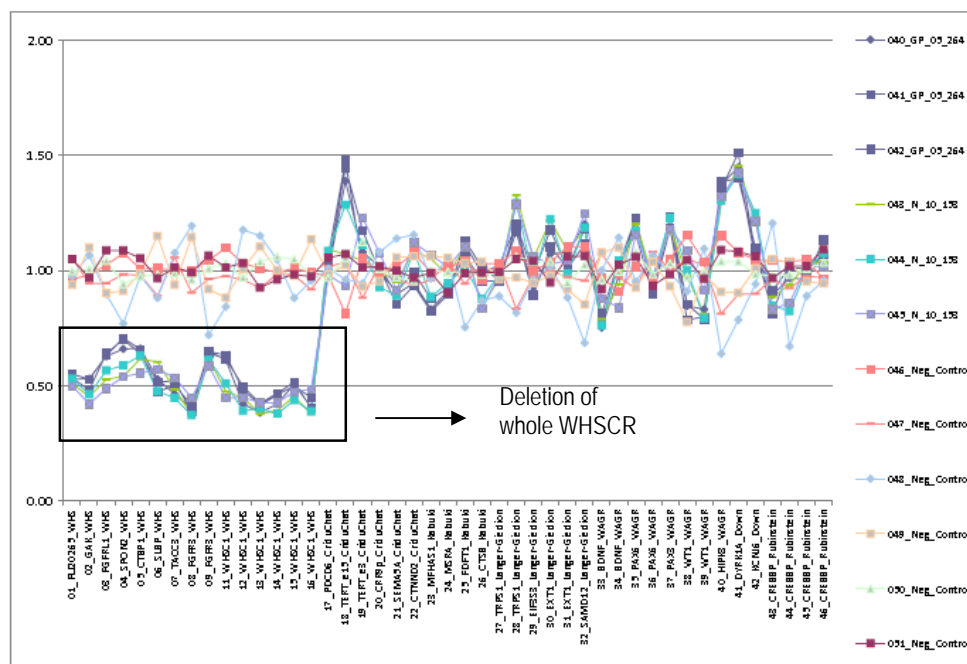


Figure 7. MLPA result using p096 probe-kits (Syndromatic kits) of subject N-10

Deletion of whole WHSCR region (4p16.3) is identified in this subject sample using MLPA with probe-kits P096.

V.2.2. Subject 2 (Dup 2pter)

Wakita et al. reported a subject with a duplication of 2p25.1-25.3 with a minimal phenotype. The phenotypic features included hypertelorism, an abnormal shape and position of the ears and long and hyperextensible fingers⁶⁶. However,

Al-Saffar et al. reported a larger terminal duplication of 2p involving band 2p23pter with a more distinctive phenotype comprising a prominent forehead, a depressed nasal bridge, posteriorly rotated ears, generalized hypotonia and a delayed fine and gross motor development ⁶⁷. Some clinical manifestations of this Subject are similar to the Subject reported by Saffar et al., as indicated in Table 8. Based on the clinical features, this subject is likely to have true 2p duplication since his phenotype showed several similarities with the previously reported cases.

Cytogenetic analysis confirmed this abnormality [This patient had karyotype of 46,XY,add (2)(p25)] . Therefore in the future FISH is needed to see the location of the duplication, Parental testing is needed to see whether the duplication is de novo or inherited. Then SNP array is warranted also to measure the exact size of duplication.

V.2.3. Subject 3 (Del 18 pter)

Microscopically visible 18p deletions have been reported in numerous cases ¹¹. Furthermore, familial microscopically visible 18p deletions have been reported as well ^{68,69}. Nevertheless, none of these describe a sub-microscopic deletion of 18p11.3pter. Horsley et al. suggested that such small 18pter deletions are only associated with some degree of mental retardation. However, in addition to the mental retardation several distinctive dysmorphological features were noted in this Subject as indicated in Table 8 ⁷⁰. Some of these features are similar to the most consistent features caused by visible deletions of 18p, such as microcephaly,

hypertelorism and a low nasal bridge⁷¹. Based on the clinical features of this subject we presume she might have a large, microscopically visible deletion. However the cytogenetic analysis showed a normal karyotype (46,XX). Therefore further analyses (FISH and SNP array) are needed.

V.2.4. Subject 4 (Dup 16qter)

Maher et al reported three cases with 16q duplication. All these reported cases resulted from a parental translocation. Therefore it is difficult to compare the phenotype of pure 16qter duplication with the literature because some of the described features in these reported cases are caused by the coexistence of a partial autosomal monosomy⁷². In summary, frequently noted features were mental retardation, growth retardation, a high forehead, a beaked nose, a long philtrum and a micropenis in males. In this Subject, only mental retardation and a high forehead were similar with the previous reported cases. In general, subtelomeric duplications only if they are small are more difficult to confirm than deletions and therefore this Subject needs to be confirmed with another analysis before the definite results are complete. Nevertheless, we assume this duplication will be confirmed because both the P070 and P036D probes identified the same duplication and the subject showed a few similar clinical features with previous reported cases, as noted in Table 8.

V.2.5. Subject 5 (Del 10qter / dup 9pter)

De Vries et al. reviewed 20 subjects with a 10q26.1qter deletion and concluded that all subjects have a consistent phenotype including mental disability¹¹, growth retardation, microcephaly, a triangular face, hypertelorism, strabismus, a prominent nasal bridge, low set ears, micrognathia, a short neck, cryptorchidism, anal/urogenital, cardiac and renal anomalies. Overlapping features of this subject included: mental retardation, strabismus, low set ears, micrognathia and a short neck. In addition to the 10qter deletion 9pter duplication was identified with the P070 and P036D probe-sets. Therefore, this subject may also have some characteristics of a duplication of 9pter. Although several cases of duplication 9p were reported pure 9p terminal duplication are rare. Only three subjects with a duplication of 9p22p24 have been previously reported⁷³. Clinical manifestations in those cases included MR, brachycephaly, wide-spaced eyes, apparently low set ears, down-turned corners of the mouth, and a mild hand anomaly. This subject had some similar clinical features such as MR, brachycephaly and low-set ears. Interestingly, this subject had a unilateral cleft lip also which has never been reported in both 10qter deletions and 9pter duplications. Based upon the MLPA results and the comparison to previous reported cases we suggest that the clinical features of this subject are caused by the 10qter deletion and the 9pter duplication. Due to the presence both of deletion and duplication in this subject, unbalanced translocation is thought. Therefore cytogenetic result is needed to confirm microscopically visible unbalanced translocation (to see whether it is present or not). However in the cytogenetic analysis there was a normal karyotype (46,XX). We suggest that the translocation is very small, thus FISH and/or array

is needed for further assessment. In addition, parental testing is needed to trace the balanced translocation in the parent.

V.2.6. Subject 6 (Del 18pter / Dup 4pter)

As discussed in Subject 3 an 18p deletion leads to a wide range of associated clinical features ranging from MR as the single manifestation to MR with severe dysmorphisms depending on the size of the deletion. Several phenotypic features in this subject, as indicated in Table 8, were similar to the most consistent microscopically visible 18p deletion features such as the microcephaly, the ptosis, the low nasal bridge, wide-spaced nipples, a clinodactily of the fifth fingers and strabismus⁷¹. In addition to the 18pter deletion, this subject has a 4pter duplication and therefore might also have characteristics of 4pter duplication. Takeno et al. reported a family with terminal 4pter duplication cases⁷⁴. Most consistent features were a severe or moderate mental retardation with behavior problems and a minor phenotypic alteration. Furthermore, Rodriguez et al. reported a case with a 4pter duplication without mental retardation and distinctive clinical features except with unusual reproductive history⁷⁵. In conclusion, both the clinical features of this subject and the MLPA results support the fact that her features are caused by a deletion of 18p. Unfortunately, in this Subject the cytogenetic analysis was not finished yet. Therefore the actual size of the deletion was not identified. It is difficult to conclude whether some of the features in this subject are caused by the duplication on chromosome 4pter since the clinical features of such duplication are only MR with behavior problems and minimal phenotypic characteristics.

Due to the presence both of deletion and duplication in this subject, unbalanced translocation is thought. Therefore cytogenetic result is needed to confirm microscopically visible unbalanced translocation (to see whether it is present or not). However in the cytogenetic analysis there was a normal karyotype (46,XX). We suggest that the translocation is very small, thus FISH and or array is needed for further assessment. In addition, parental testing is needed to trace the balanced translocation in the parent.

V.2.7. Subject 7 (Dup 9pter)

Duplications of chromosome 9pter were discussed in Subject 5. However, in this subject it is only 9pter duplication without additional deletion like in Subject 5. Some phenotypic features of 9pter duplication, as reported by Franchi et al., were also noted in this subject (Table 8) ⁷⁶. He had deep-set eyes, apparently low set ears and mild hand anomalies. Since some similarity with the previous reported case was noted in this subject and both of the STDs probes gave a similar result we presume that the clinical features in this subject are caused by terminal 9p duplication. Cytogenetic result was normal (46,XY). However, further confirmation (SNP Array) is necessary whereas duplications are more difficult to prove compared to the deletions.

V.2.8. Subject 8 (Del XYqter)

No pure submicroscopic Xqter deletion has been reported ¹¹. However Bates et al reported microscopically visible Xqter deletion in female patient with

ovarian failure ⁷⁶. This subject was noted to have distinctive clinical features as indicated in Table 8. Due to a minimum amount of previously reported cases with Xqter subtelomeric deletion, it is difficult to compare with previously reported cases. Interpretation of this result is also difficult since the Xqter probe (P070 kit) is located in the Pseudo Autosomal Region (PAR 2 Region). This region consists of 800 Kb identical DNA sequences at the q telomeric ends of the X and Y chromosomes, called the pseudoautosomal region 2 (PAR2); another region of approximately 2500 Kb identical DNA sequences at the p telomeric ends of these chromosomes (PAR1).

Repetition of the analysis, using the P070 probe kit again, showed the same result. Since the deletion of this region does not explain the phenotype, we presume it might be due to a large Xq deletion or something in Y-chromosome. Furthermore, the cytogenetics result is normal (46,XY), therefore FISH and SNP-array are needed.

V.2.9.Subject 9 (del 4q / dup 10 q)

There were 2 Subjects with the same aberration; however they have different clinical features (Table 8). Probe P036C was used initially in these samples was repeated two times and showed the same results. Reanalysis with P070 probe not confirmed the results. Possibly these results were caused by segmental duplications. Furthermore, this result indicated that the 10q probe in the P036C can detect different copy numbers. Due to different clinical features in those subject and the result of probe P036C was not confirmed by P070.

Polymorphisms were thought to explain this result because sequencing in these samples which is surrounding the probe 4q was undertaken also. However, no polymorphisms were found. Cytogenetics analysis was showed normal karyotype in both subjects (46,XY)

V.2.10. Subject 10 (dup 21 pter/qter)

When the MLPA analysis with probe P070 identified a duplication of 21 p and 21q a trisomy 21 (Down Syndrome) was suspected. Further, cytogenetic analysis confirmed this result. There was a clinical suspicion before.

V.2.11. Subject 11 (Dup 10pter)

In the initial analysis, using the probe P070, 10pter duplication was identified. However, reanalysis using the P036D probe did not confirm this duplication. The clinical features of this subject were compared to a similar 10pter duplication case in the ECARUCA database (case no 4224; www.ecaruca.net). However, except for the mental retardation, there were no overlapping clinical features. This patient had a normal karyotype (46,XY). The duplication could be due to a polymorphism although a very small duplication is still possible. Therefore, further analysis (SNP array) is warranted.

V.2.12. Subject 12 (Dup 10qter)

Duplications of chromosome 10q are recognized as a distinct syndrome. Numerous cases have been reported, and in most cases, the aberration is a

consequence of a familial balanced reciprocal translocation. Terminal duplications of 10q were considered to cause more severe features than proximal duplications (Han et al. 2004). In the initial analysis with the P070 probe 10qter duplication was identified. However, this result was not confirmed by reanalysis using the P036D probe. In this Subject mild characteristic features were noted in contrast to severe multiple congenital anomalies reported in 10qter duplication cases by Han et al.⁷⁷. This patient had a normal karyotype (46,XY). The duplication in this Subject is might be due to a polymorphism although a very small duplication is still possible. The phenotype in this Subject did not similar with 10q duplication syndrome that has been described before. Therefore SNP array could help to know how big the deletion is

V.2.14. Prevalence of STDs in the population

It is difficult to calculate the actual prevalence of sub-telomeric chromosomal abnormalities in the Indonesian population before all identified cases are confirmed. However, the fact that several samples showed a sub-telomeric abnormality confirmed that STDs are one of the causes of mental retardation in this population. If the prevalence is calculated based on the initial screening with probe P070 the prevalence of STDs in this population would be 8,5 % (10/117). The prevalence would decrease to 5,1 % (6/117) if only the cases with a confirmed aberration, shown by both P070/P036D probe-sets including one case with consistent results after confirmed with P070 (sample with deletion in XYp), would be considered. Koolen et al. proposed parental testing and a clinical

comparison with previously reported subjects with similar subtelomeric rearrangements, in order to exclude polymorphisms¹². In this study parental testing was not available yet but all cases were clinically compared to the available literature cases. Based on these findings, only subjects with overlapping clinical features compared to the reported cases should be counted. In that case, the prevalence would be 4,3% (5/117). The latter seems to be ‘a reasonable’ prevalence because it was calculated based upon both the confirmed MLPA results and the clinical features present in the subjects although microscopically visible translocations still cannot be excluded. Translocations are thought of since 2 samples were detected which had both of a deletion and duplication. In addition, this prevalence is comparable to the prevalence of 5,4% found in the Dutch population¹². Similarly, Rooms et al. found 5,3% in the Belgian population¹⁶. The prevalence of STDs in this research is almost consistent with those studies in Caucasian population.

V.3. PWS/AS result

Only subjects with clinical signs of PWS/AS were screened for these syndromes. However, none of them showed a positive result. Jacobsen et al reported a high prevalence of AS among the mentally retarded 1,4% (4/285) in contrast to Vercesi et al who reported a very low prevalence of PWS / AS in their cohort (0/256)^{78,79}. However, the latter study calculated that there was no statistically significant difference between their prevalence number and Jacobsen’s. Several community based studies reported the prevalence of PWS is

1:25.000 and of AS is between 1:10.000 – 1:40.000 respectively⁷⁹⁻⁸⁴. Although PW/AS was not detected in our cohort, our result is overlapping with the result as studied by Vercesi et al. In addition, tested cohort in the Indonesian population is smaller than studied by Verseci and Jacobsen. For future research it has to be considered to use a larger cohort of samples to test for PWS/AS.

V.4. Cytogenetics result

Cytogenetics analysis in this study only showed one subject with trisomy 21. Although the clinical examinations were performed very careful to exclude subject with suspicion of Down Syndrome, nevertheless we found one subject with trisomy 21 and two subject with different structural abnormalities.

Two subjects were identified to have structural abnormality. One subject with duplication of 2p25 was confirmed by MLPA. One subject with deletion of 4p was confirmed to have deletion 4p and duplication of 8p. Further analysis is needed to confirm MLPA result.

Rauch et al. reported the highest prevalence of trisomy 21 (9,2%) among other chromosomal aberrations within the mentally retarded population⁸⁵. However, the total prevalence of all microscopic visible chromosomal aberrations, including trisomy 13, trisomy 18 and other autosomal aberrations was reported to be 5,2 %. Down syndrome is excluded in this study, thus only small percentage of trisomy detected by our cytogenetics analysis.

The differences between the result and with another studies may result from difference of population studied (mild-moderate MR) compared to the reference (general MR population).

V.5. Clinical examination protocol

The standardized clinical examination protocol adapted from the RUNMC was very helpful to describe the dysmorphological features in the subjects and it offers a systematic approach to use in a clinical setting. However, it has to be taken into consideration that in some cases not all steps of the protocol could be completed. Good anamneses are difficult to carry out and often there is no complete medical record of the subjects. This might be caused by a lack of education and knowledge of the parents about the medical history and the motor milestones of their child. However, by using this standardized protocol the clinical examination of the subjects was more easily performed.

V.6. Clinical Studies

Dysmorphological assessments of MR subjects are helpful to establish genetic diagnosis. This study showed that one subject with a full expansion of the CGG repeat had major clinical sign of FXS i.e. mental retardation, shyness behavior, a long face, a pointed chin, long fingers and macroorchidism. In the clinical setting, these features could help the clinician to raise susceptibility of Fragile-X syndrome, if they found MR individuals with those clinical features.

In order to discuss the clinical features among MR individuals with subtelomeric rearrangements, the discussion will be focused only in the MR individuals who had a positive subtelomeric rearrangement in both STD probe-kits (5 subjects). Subjects with microscopically visible subtelomeric rearrangements were excluded (2 subjects).

Microcephaly was found in 3 out of 5 subjects (60%). Eye abnormalities, including telecanthus, hypertelorism and ptosis were found in 5 out of 5 subjects (100%). Ears abnormalities including ears structure and position abnormality were found in 3 out of 5 subjects (60%). Nose abnormalities were found in 3 out of 5 subjects (60%). Mouth area abnormalities were found in 5 out of 5 subjects (100%). Extremities abnormalities were found in 4 out of 5 subjects (80%) (See Table 1 in Appendix 8). Based on common features observed in subtelomeric rearrangements cases, the common clinical features in these subjects are microcephaly, ears, eyes, nose, mouth and extremities abnormalities. In clinical setting those clinical features should raise susceptibility of subtelomeric rearrangements if we found those features in individuals with MR. Furthermore, a study have been done by de Vries et al, 2001 suggested using their clinical check list to screen subtelomeric rearrangements in individuals with MR⁸⁶. Thus, for the next study, de Vries checklist for subtelomeric screening may be considered.

CHAPTER VI

CONCLUSION AND FUTURE DIRECTIONS

VI. 1. Conclusion

The prevalence of FXS in this cohort was 1,65 % (2/121) in the whole population and 2,15 % (2/93) in the male population. Although not all STDs results could be finalized yet, “a reasonable” prevalence of STDs in the population was calculated to be 4,3% (5/117) however, translocations are thought in 2 samples and therefore this still have to be confirmed in some cases by further analysis. The prevalence of STDs also overlapped with a study in the Caucasian population ^{11,15}.

None of the selected cases in this cohort had PWS or AS and therefore it can be preliminarily concluded that the frequency of PW/AS is very low in this population. The low prevalence for PW/AS found in this cohort was similar with the previously reported prevalence in Caucasian population excluding Jacobsen’s study ⁸⁰⁻⁸⁴. The prevalence of microscopic visible aberrations in this population is (3/ 122).

The prevalence of FXS in the male population was similar than previously reported by Faradz et al. and the similar too (~ 2 — 3%) as reported in the Caucasian population ^{6,87-90}

1. This research confirmed that the prevalence of STDs is higher than Fragile-X and become major genetic abnormalities causing mental retardation in this population, excluding Down Syndrome and other

microscopic visible cytogenetic abnormalities. The results presented in this report represent the major genetic causes of MR in Indonesia, excluding Down Syndrome. This is the first screening study for STDs and PW/AS in the Indonesian population. Despite some of STDs results still need to be confirmed with further analyses we proved that STDs are a major cause of MR in the Indonesian population.

2. There was an association between genotype and phenotype of the affected individuals and each association were discussed in discussion section.

VI.2. Future Directions

In next studies in the same field, a good planning and scheduling, particularly in time and financial support, has to be considered. Also, the right orders of laboratory assessments, as written in the protocols, have to be followed correctly. The results of this research project should raise the level of awareness concerning the existence of important genetic causes of MR in the Indonesian community. Furthermore the results of this research can be used as a basis for genetic counseling in the Indonesian population.

SUMMARY

Mental retardation (MR) is still a major health problem in all countries including the developed countries. Some etiologies of MR are known, like biochemical causes, chromosomal abnormalities, mutations of single gene (Mendelian disorders/mitochondrial disorders), multi-factorial disorders or because of environmental factors such as toxins, infections, and trauma. However, genetic factors have a crucial role since approximately half of MR cases have a familial history

The knowledge of genetic factors of mental retardation by cytogenetics, molecular and clinical assessment is an advantage in giving early diagnosis and prevention through genetic counseling to subject's family. These serve as diagnostics tool in determining genetic factor that may play role as etiological cause of MR. Nowadays, the above mentioned assessments could well be done in Indonesia. But due to lack of facilities in Indonesia, further molecular assessments have to be carried out in other centre abroad.

Up till now there are only few studies on MR in Indonesian population carried out by Indonesian researchers or in collaboration with researchers abroad. No MR protocol has been applied for diagnosis of MR in Indonesia, thus this study aims to establish MR protocol for diagnosis in Indonesia. More over genetic assessments as an etiological diagnostic tool for MR have not yet been recognized as a routine

diagnostic tool in Indonesia. The procedures for genetics assessment will be the main focus of this research in order to apply this MR protocol for diagnosis in Indonesia.

The main research question is what the percentage of genetic factors playing a role in the etiology of mental retardation in Indonesian Population. The advantages comes out from this research will be encouraging public awareness of genetic diseases. Furthermore, the advantage of this research will be laying down a basis for genetic counseling. Genetic counseling is not that common in Indonesia, though with increasing public awareness in genetic diseases there will be an increasing demand for genetic counseling.

Mental retardation (MR) refers to substantial limitations in present functioning. It is characterized by significant subaverage intellectual functioning i.e $IQ < 70$, existing concurrently with related limitations in two or more of the following applicable adaptive skill areas: communication, self-care, home living, social skills, community use, self-direction, health and safety, functional academics, leisure and work. The age of onset should be before age 18.

Many environmental and genetic factors can cause MR like biochemical causes, chromosomal abnormalities, mutations of single gene (Mendelian disorders/mitochondrial disorders), multi-factorial disorders or because of environmental factors such as toxins, infections, and trauma. Nevertheless, genetic factors have a crucial role since approximately half of MR cases have a familial history.

Several genetic causes of MR are known, most common are the large microscopic numerical and structural cytogenetic abnormalities (> 4 MB), such as trisomy 21 (Down syndrome), large deletions and duplications and unbalanced reciprocal and Robertsonian translocations. Down syndrome is the best known chromosomal abnormality as cause of MR with the highest prevalence. This abnormality occurs due to meiotic non-disjunction, mostly maternal with a result an extra 21 chromosome in the child. With routine chromosomal analysis, trisomies like trisomy 21 can be easily detected.

Individuals with MR, dysmorphic features with or without positive family history (usually without positive family history) and without microscopic chromosomal abnormalities can be due to cryptic chromosomal rearrangements like sub-microscopic sub-telomeric deletions or duplications. This has been identified as another common cause of MR.

X-Linked Mental Retardation (XLMR) can be generally classified into two categories; syndromic XLMR (S-XLMR) that are associated with a specific or characteristic phenotype and non-syndromic XLMR (NS-XLMR) that do not present with consistent clinical features. Non-syndromic XLMR was thought to be more frequent than the syndromic XLMR conditions. Nevertheless, due to the development of molecular techniques which is used to classify family and better detailed examination in the patient, the proportion of S-XLMR will be increase simultaneously with the decrease of NS-XLMR.

Fragile-X syndrome is one of the XLMR and become the most common inherited abnormality causing MR. This syndrome is commonly seen in males. However, females can also be affected. Approximately 50-60% of female full mutation carriers will have borderline to mild mental retardation. The Fragile X syndrome is due to a mutation in a gene on the X chromosome leading to hyper-expansion of a trinucleotide (CGG) repeat sequence. There is a repetitive CGG sequence on the 5'UTR of the *FMRI* gene and normal individuals have approximately 5 to 44 CGG repeats. There is a gray zone of 45 to 54 repeats that can be associated with minor instability from generation to generation. The premutation is defined as 55 to 200 repeats and the full mutation of the *FMRI* gene involves an expansion of >200 CGG repeats and is clinically associated with features of Fragile-X syndrome.

Prader-Willi and Angelman syndrome are genetic disorders that show different levels of mental retardation. Prader-Willi syndrome (PWS) features mild to moderate mental retardation and Angelman syndrome (AS) severe mental retardation. These syndromes can occur due to absence of the paternally or maternally derived chromosome 15q11-13 region by several genetic mechanisms. When there is absence of paternal chromosome 15q11-13 region it will manifest as Prader-Willi Syndrome, while absence of the maternal chromosome 15q11-13 region will manifest as Angelman syndrome.

Approximately 70% individuals with PWS and AS have a deletion of one chromosome 15 involving bands 15q11-q13. Whereas in PWS uniparental maternal

disomy occurs in about 25% of cases, in AS uniparental paternal disomy occurs in only 1% of cases. Both syndromes and in particular AS can also be caused by mutations in imprinted genes with potentially high recurrence risks.

II.1.3 Genetic diagnosis

Genetics diagnosis for MR individual is based on dysmorpological and laboratory assesement. Most known genetics syndrome in MR is associated with specific features¹⁰. Laboratory assessment is considered as the tool to confirm the genetics diagnosis based on clinical examination. Nevertheless, some genetics etiology could not be diagnosed with dysmorphological assessment. Thus, laboratory assesement is needed. However, dysmorphological assessment is the most important thing to do before laboratory and other assessment.

Chromosomal analysis is the most valuable analysis to reveal genetics cause of MR. With routine chromosomal analysis, numerical abnormalities such as trisomy 13,18, and 21 or monosomy such as monosomy X (45,X/Turner syndrome) can be easily detected. Furthermore chromosomal analisys can reveal some structural chromosome abnormality such as deletion / duplication (> 4 Mb), insertion, derivation, and translocation. That is why chromosome analysis should be done as the first laboratory assessment for the patient.

Since Fragile-X syndrome is considered as the most common inherited abnormality causing MR. Laboratory analysis for the Fragile-X syndrome is the most significant assessment after chromosome analysis. Although fragile site in the Fragile-X syndrome is detected in chromosomal analysis, nevertheless subject with

permutation or mosaic permutation – full mutation of Fragile-X syndrome might not be detected by routine chromosome analysis. That is why confirmation analysis should be done with molecular assessment.

Subtelomeric deletions and duplications ($< 4\text{Mb}$) are not visible by microscope. Thus molecular analysis of this abnormality is required. Based on the high incidence of this abnormality in MR individual, molecular screening of this abnormality is very important if the chromosome analysis and Fragile-X analysis fail to detect the genetic cause of MR.

PW/AS analysis could be performed to the MR individual with suspicion of this abnormality. Since there are some genetic diseases have similar features with PW/AS syndrome, this analysis is valuable to verify genetic diagnosis of MR especially to the MR individuals with suspicion of this syndrome.

This study is an observational survey. Pupils have been examined from several special schools for the mentally retarded in Semarang namely SLB Negeri, SLB Pelita Ilmu, and SLB Hj. Soemijati. Pupils with a known exogenic cause, such as pre-, ante- and postnatal traumas, or a history of asphyxia, non cooperative pupils and children of parents who declined to sign the informed consent form, were excluded from research participation. Also subjects with a clinical suspicion of Down Syndrome.

Routine chromosome analysis was performed in Molecular and Cytogenetic laboratory of Center for Biomedical Research, Faculty of Medicine Diponegoro University Semarang. DNA analysis of the Fragile-X syndrome, Prader-

Willi/Angelman syndrome and sub-telomeric deletions and deletions (STDs Analysis) with MLPA (Multiplex Ligation Probe Amplification) were performed in the Radboud University Nijmegen (Laboratory for DNA diagnostics, RUNMC).

Samples were taken from 122 mentally retarded pupils with an average age between 6 and 25 years. The parents were also involved in order to obtain the family history. Furthermore, samples of parents, of pupils with a proven genetic abnormality, were taken in order to determine *de novo* or inherited occurrence. Not all parents were available for sampling.

After a clinical examination 5 mL heparinized blood (for chromosome analysis) and 5-10 mL EDTA blood (for DNA analysis) were drawn from each individual. Chromosome preparation, chromosome analysis and DNA Isolation were performed following the standard operating procedure of Center for Biomedical Research (CEBIOR) Semarang.

CGG repeats in *FMR-1* were amplified with PCR technique. Southern blot was used to confirm the CGG results. MLPA is a new method for relative quantification of about 40 different DNA sequences in an easy to perform reaction. This technique has been developed and first described by MRC-Holland. To detect STDs in samples, P070 probe-kit and P036D was used using MLPA analysis. MLPA is a new method for relative quantification of about 40 different DNA sequences in an easy to perform reaction. This technique has been developed and first described by MRC-Holland. To detect PW/AS in the samples P028 methylation specific probe-kit

was used. All procedures mentioned above were performed following a standard operational protocol from the Department of DNA Diagnostics of the RUNMC.

From 186 pupils selected in three special schools, only 122 pupils can be included in further analysis based on the inclusion and exclusion criteria.

A total of 122 subjects with developmental disability from three special schools in Semarang were analyzed for trinucleotides repeats expansion (PCR Analysis on agarose and genescan). To determine exact number of CGG repeats, PCR products were analyzed by Fragment Length Analysis (genescan). Southern Blotting was used when the PCR / Fragment Length Analysis were not clear; there was no PCR product in agarose gel (1 sample), or when the CGG repeats were too high (1 sample) and when single allele (can be homozygous samples or normal and expanded allele) found in woman samples (9 Samples). Southern Blot analysis was performed in those samples which identified one subject with a full CGG expansion, one subject with a mosaic pattern (premutation and full mutation) and none of the females were found to have a CGG expansion. The CGG repeat from those samples then redistributed. The most frequent CGG repeat in this population was 29 CGG repeat, then followed by 28,30,35 & 36 respectively.

To identify sub-telomeric deletions and duplications (STDs) analysis by MLPA was carried out. One male was excluded for STDs analysis because of a positive FMR-1 result. However one sample cannot be analyzed using MLPA analysis due to bad DNA. Therefore 120 samples were tested with MLPA analysis. The initial screening with the P070 kit (MRCH) which contains probes in all

telomeric regions of the different chromosomes showed 13 samples with subtelomeric rearrangements. Re-analysis using another probe-set (P036D) by DNA Diagnostic RUNMC confirmed that 8 out of 13 indeed had a sub-telomeric rearrangement. One sample was excluded from further analysis after the result of MLPA was confirmed with cytogenetics showing trisomy 21 (Down syndrome). Three samples that did not showed same aberration by confirmation with P036D probe were considered most likely because of polymorphism but it still could be something (very small deletion / duplication).

The family of the proband with a full CGG expansion was visited and blood samples were taken from all family members in order to detect carriers and possibly affected individuals. Several carrier females were identified in the family; the mother had a normal allele and an elevated band size in PCR amplification which was confirmed by Fragment Length Analysis and Southern Blot. The first daughter had 1 normal allele and 1 allele in the premutation range. Two brothers of the proband had a mosaic premutation allele which was assessed by PCR and fragment length analysis and confirmed by Southern Blot. The second daughter had normal alleles. The youngest daughter showed 1 normal allele and 1 fully expanded allele which was assessed by Southern Blot. Except for the affected proband all sibs and the carrier mother apparently had a normal intelligence. However, one male had learning difficulties.

Cytogenetic preparations were carried out in all samples; One sample with trisomy 21 was detected, two with structural abnormalities [46,XY,del(4)(p16) and

46,XY,add (2)(p25)] and the others showed normal karyotype. Only subjects with clinical signs of PWS/AS were screened for these syndromes. However, none of them showed a positive result.

The standardized clinical examination protocol adapted from the RUNMC was very helpful to describe the dysmorphological features in the subjects and it offers a systematic approach to use in a clinical setting. By using this standardized protocol the clinical examination of the subjects was more easily performed.

In the MR individuals who had a positive subtelomeric rearrangement in both STD probe-kits (5 subjects), microcephaly was found in 3 out of 5 subjects (60%). Eye abnormalities, including telecanthus, hypertelorism and ptosis were found in 5 out of 5 subjects (100%). Ears abnormalities including ears structure and position abnormality were found in 3 out of 5 subjects (60%). Nose abnormalities were found in 3 out of 5 subjects (60%). Mouth area abnormalities were found in 5 out of 5 subjects (100%). Extremities abnormalities were found in 4 out of 5 subjects (80%).

The prevalence of Fragile-X Syndrome (FXS) in this cohort was 1,65 % (2/121) in the whole population and 2,15 % (2/93) in the male population. Although not all STDs results could be finalized yet, “a reasonable” prevalence of STDs in the population was calculated to be 4,3% (5/117). The prevalence of STDs also overlapped with a study in the Caucasian population.

None of the selected cases in this cohort had PWS or AS and therefore it can be preliminarily concluded that the frequency of PW/AS is very low in this

population. The low prevalence for PW/AS found in this cohort was similar with the previously reported prevalence in Caucasian population excluding Jacobsen's study.

The prevalence of FXS in the male population was similar than previously reported by Faradz et al. and being similar too as reported in the Caucasian population

This is the first screening study for STDs and PW/AS in the Indonesian population. Despite some of STDs results still need to be confirmed with further analyses we proved that STDs are a major cause of MR in the Indonesian population.

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Appendix 1. Figure of FMR-1 Amplification result on agarose and genescan from patients SM-30C and SM-34C

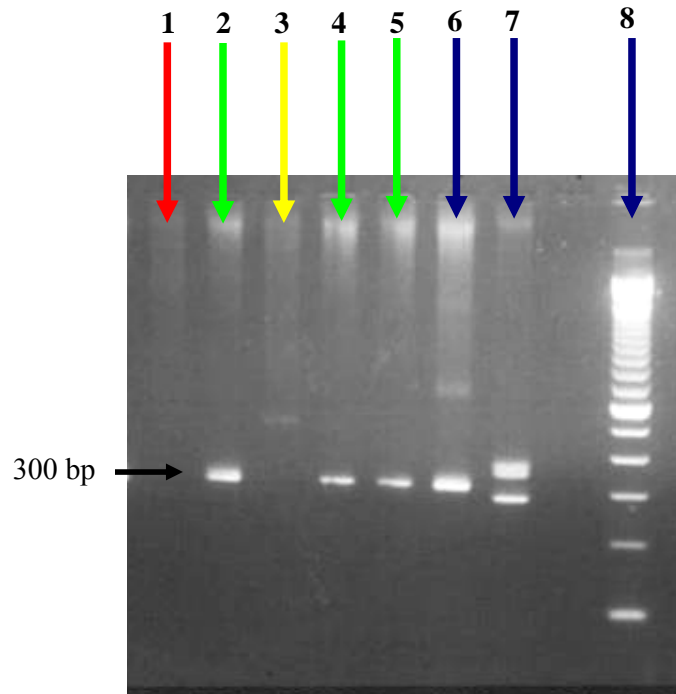


Figure 1A. Agarose gel of FMR-1 amplification from SM-30C and SM-34C

Lane 1 (SM-30 C) no PCR product was detected

Lane 3 (SM-34 C) showing high CGG repeats / premutation allele (~500 bp)

Lane 2,4,5, are male normal subjects

Lane 6 is premutation female control

Lane 7 is normal female control

Lane 8 is marker

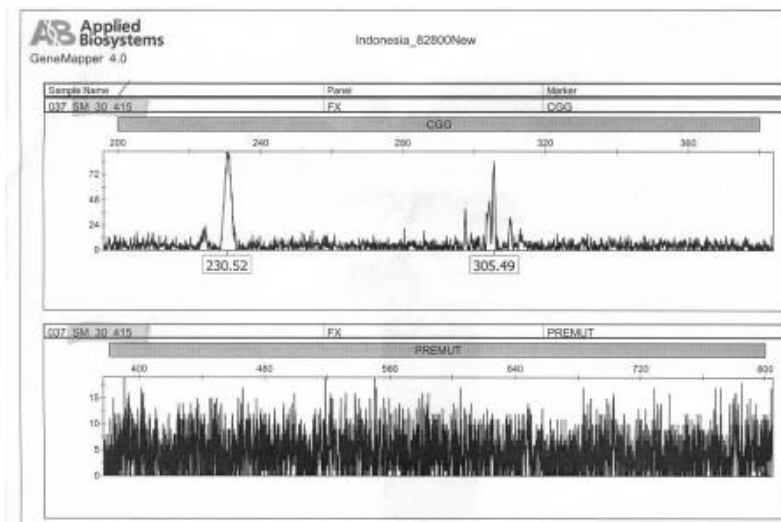


Figure 1.B. Genescan result from SM-30 C

There was no peak detected by Fragment Length Analysis in this subject. The peak on the 230 CGG repeats and 305 repeats are artifact. This confirms that there is no PCR product.

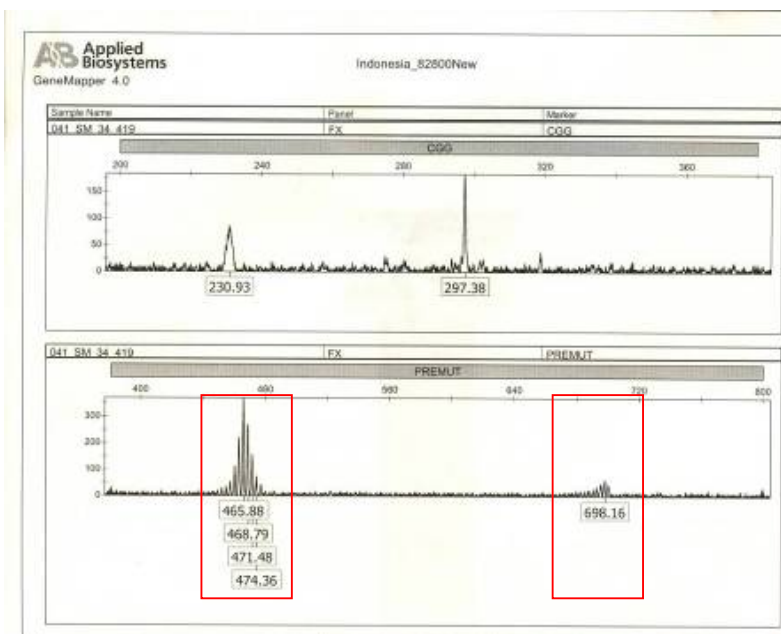


Figure 1.C. Genescan result from SM-34 C

Peaks were detected on ~468 CGG repeats and 698 CGG repeats. This analysis confirmed that there were mosaic permutation alleles in this subject.

Appendix 2. Figure of Southern Blot analysis result of SM-30C, SM-34C and some homozygous females

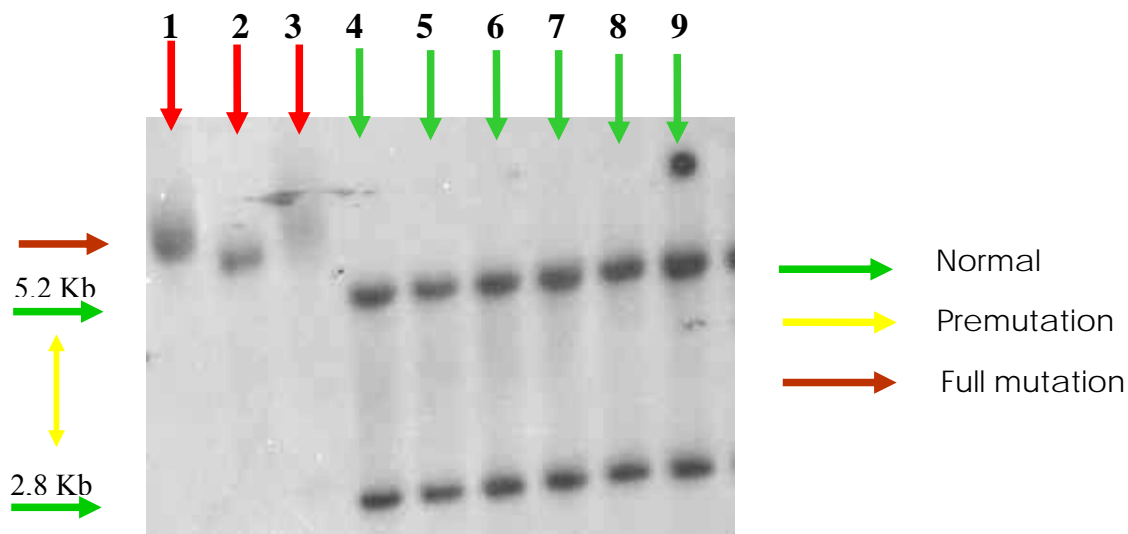


Figure 2. Southern Blot analysis result of SM-30C, SM-34C and some homozygous females (correspond to table 6).

Lane 1 (SM-30 C) Full mutation; Showing expanded allele

Lane 2 (patient not in this population) Full mutation; Showing expanded allele

Lane 3 (SM-34C) Mosaic premutation to full mutation; Showing expanded allele (>5,2Kb) and slight smear above the unmethylated allele (2,8 Kb).

Lane 4-9; (4; N-52), (5; N-54), (6; N-52), (7; PI-19), (8; PI-21), (9; SM-20): Showing two normal female alleles

Work with a methylation sensitive enzyme allows us to distinguish between methylated and unmethylated *FMR1* alleles. Normal female samples (Lane 4-9) showed clear band of 2,8 kb and 5,2 kb correspond to unmethylated normal allele and methylated normal allele due to X-inactivation process respectively. Full mutation samples showed an expanded smear correspond to high CGG repeats. Lane 3 showed expanded allele and slight smear above the unmethylated allele means that there was a mosaic pattern of permutation to full mutation.

Appendix 3. Figure of Southern Blot analysis on family members from SM-30C

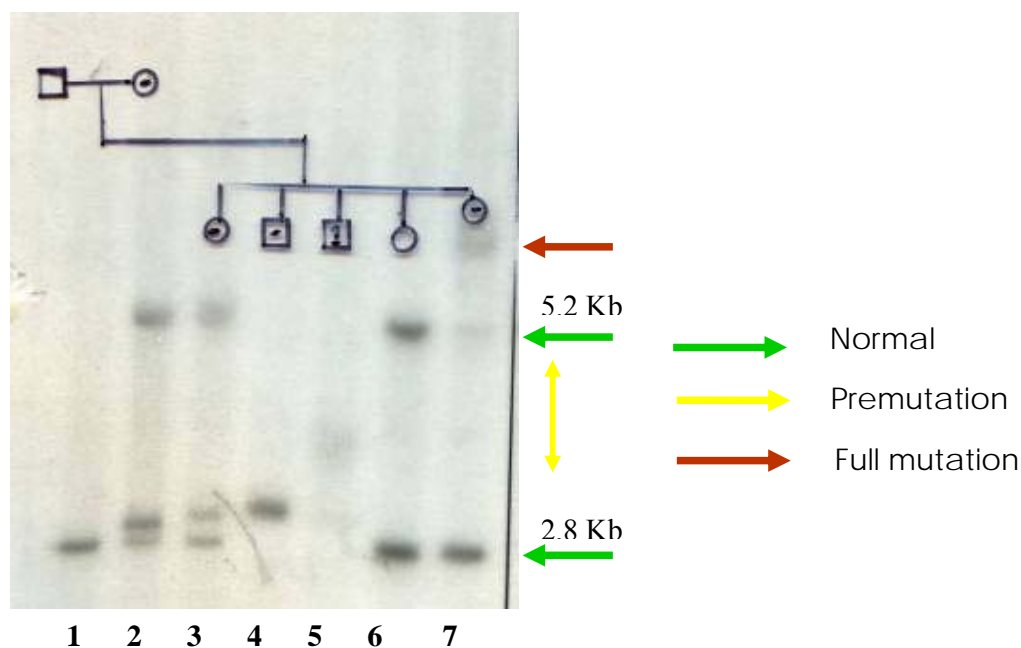


Figure 3. Southern Blot analysis of family members from SM-30C (correspond to Table.9)

Lane 1; Father; Normal allele
 Lane 2; Mother; showing normal allele and premutation allele
 Lane 3; Sister; showing normal allele and premutation allele
 Lane 4; Brother; showing premutation allele
 Lane 5; Brother; showing mosaic premutation allele
 Lane 6; Sister; showing two normal alleles
 Lane 7; Sister; One normal allele and one full mutation allele

Normal male sample (Lane 1) showed clear band of 2,8 kb correspond to unmethylated normal allele. Normal female sample (Lane 6) showed clear band of 2,8 kb and 5,2 kb correspond to unmethylated normal allele and methylated normal allele due to X-inactivation process respectively. Lane 7 showed a clear band of 2,8 kb correspond to unmethylated normal allele and expanded allele correspond to full mutation allele. Premutation females sample (Lane 2 & 3) showed a clear band of 5,2 kb correspond to unmethylated normal allele and higher band above 2,8 kb in premutation range. Premutation male samples (Lane 4&5) showed a higher band / smear above 2,8 kb in premutation range. Since the premutation allele is unstable, premutation allele sometimes was identified as two bands above 2,8 kb or smear as showed in lane 2,3 & 4.

Appendix 4. Karyotype of subjects with chromosome abberation

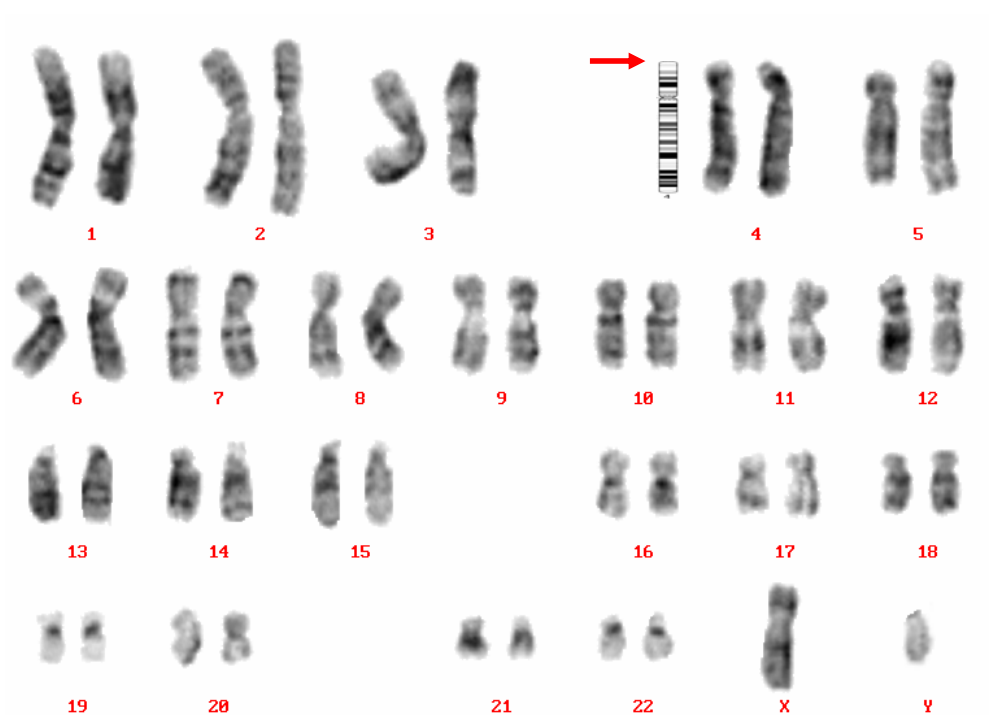


Figure 4A. Karyotype of subject N-10



Figure 4B. Karyotype of subject PI-14

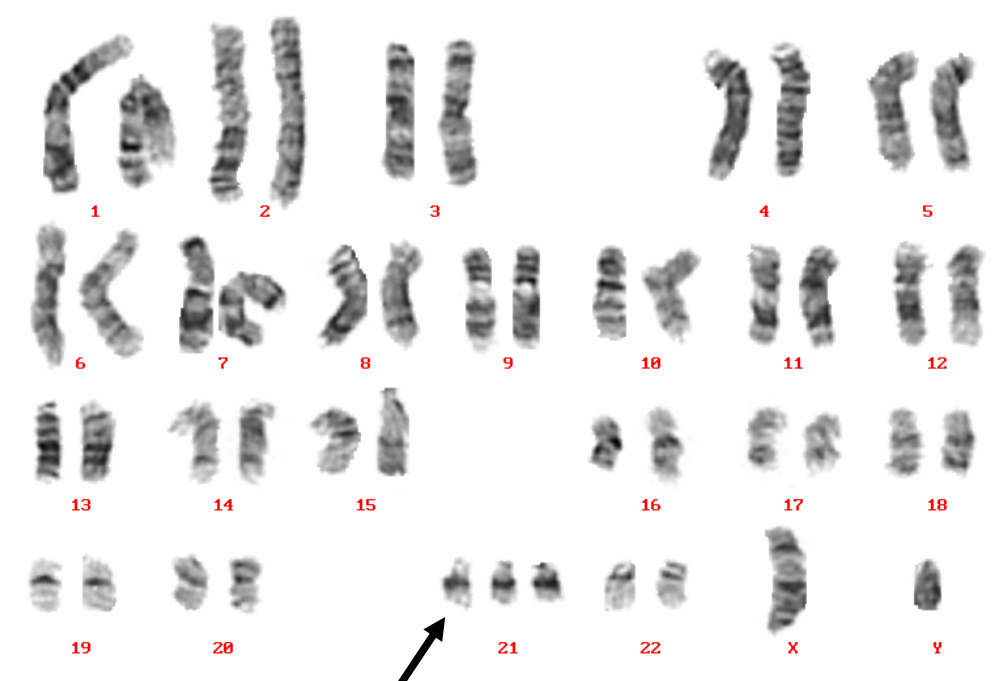


Figure 4C. Karyotype of subject N-34

Chromosome analysis with G-Banding technique showed 46,XY,del(4)(p16.3) [fig 4A], 46,XY,add(2)(p25.3) [fig 4B] and 47XY,+21 [fig 4c].

Appendix 5. Ethical Clearance

KOMITE ETIK PENELITIAN KESEHATAN FAKULTAS KEDOKTERAN UNIVERSITAS DIPONEGORO DAN RS Dr KARIADI SEMARANG

Sekretariat: Kantor PD IV-Dekanat FK Undip
Jl. dr. Sutomo 18, Semarang
Telp/Fax.024-8446905

ETHICAL CLEARANCE *No. 91 / EC/FK/RSDK/2006*

Komisi Etik Penelitian Kesehatan Fakultas Kedokteran Universitas Diponegoro/RS.Dr.Kariadi Semarang, setelah membaca dan menelaah proposal penelitian, dengan judul :

Deteksi Gen penyebab Retardasi Mental pada Populasi Retardasi Mental di Sekolah Luar Biasa (SLB) Se-Kotamadya Semarang

Peneliti Utama : dr. Farmaditya EP Mundhofir
Anggota Peneliti : 1. dr. Tri Indah Winarni,MSi.Med
2. dr. Asri Purwanti, M.Pd,Sp.A(K)

Penelitian : Dilaksanakan di 8 Sekolah Luar Biasa (SLB YPAC, SLB Swadaya, SLB Widya Bakti, SLB Pelita Ilmu, SLB Hj. Sumiyati, SLB Dharma Mulia, SLB Immanuel, SLB Negeri) Semarang dan Lab. Bioteknologi Kedokteran Undip Semarang.

Setuju untuk dilaksanakan, dengan memperhatikan prinsip-prinsip yang dinyatakan dalam Deklarasi Helsinki 1975, dan Pedoman Nasional Etik Penelitian Kesehatan (PNEPK) Departemen Kesehatan RI 2004.

Peneliti harus melampirkan 2 kopi lembar *Informed Consent* yang telah disetujui dan ditandatangani oleh peserta peneliti pada laporan penelitian.

Semarang, 27 Nopember 2006



Menyetujui,
Fakultas Kedokteran UNDIP
Dekan

Prof.dr.Kabulrachman,Sp.KK(K)
NIP.130 354 867



Komisi Etik Penelitian Kesehatan
Fakultas Kedokteran Undip/RS dr Kariadi
Ketua

Prof.Dr.dr.Tjahjono,Sp.PA(K)FIAC
NIP. 130 368 076

Appendix 6. Informed Consent

INFORMED CONSENT

Yang bertanda tangan dibawah ini:

Nama :
Orang tua dari :
Alamat :

Menyatakan kesediaan dilakukan pengambilan darah, pengambilan foto dan pemeriksaan fisik terhadap anak saya tersebut diatas untuk diteliti kemungkinan penyebab factor genetic yang menyebabkan retardasi mental/ autis tanpa dipungut biaya.

Apabila ternyata terdapat kelainan pada anak saya, saya tidak berkeberatan bila hasil-hasil tersebut (pengambilan foto, pemeriksaan fisik dan hasil laboratorium) akan digunakan sebagai laporan pada jurnal ilmiah terkait

Demikian pernyataan ini saya tanda tangani dengan kesadaran penuh dan tanpa paksaan dari pihak manapun.

Semarang,

()

Appendix 7. Physical Examination FormDr:DATE:LOCATION :

Family/proband			
Gene	Mutation	DNA/fam.nr.	Laboratory

Clinical photographs yes/no	Archived where	Consent patient/parents for use in teaching and/or scientific publications/meetings

Clinical genetic conclusion

	Diagnosis	Recurrence risk	Remarks
1			
2			
3			

Relevant patient organisation:Literature given to patient/parents:**NAME AND ADDRESS OF REFERRING/TREATING DOCTORS**

	Name	Specialism	Address
1.			
2.			
3.			

Date	Name supervisor	Signature of supervisor

PEDIGREE

Consanguinity yes/no

HISTORYConception

Exposition by profession/recreational?

Profession of man/father:

Profession of woman/mother:

Pregnancy:

fluxus

fever

skin problems

infections

trauma

toxycosis

diabetes mellitus

medicines

smoking

alcohol

X-ray/radiation

other intoxications

Prenatal care fromweeks GA by:

Prenatal diagnosis (indication and results):

Ultrasound examination (indication and results):

Delivery: by whom

where

gestational age

spontaneous

induction

artificial labour

position

duration

Apgar score

amniotic fluid

umbilical cord

placenta

W (P:)

L: (P:)

OFC:

(P:)

asphyxia

icterus

artificial ventilation:

how long in hospital:

Neonatal period:

feeding problems

hypotonia

Psychomotor development:

laughing

grasping

regression yes/no

making noises

sitting with help

rolling over

standing

walking

sitting without help

social contact

speech

behaviour

school

Past illnesses/admissions/operations

Paramedical treatment (physiotherapy, speech therapy etc.)

Prescribed medicines

SPECIFIC HISTORY

EXAMINATION

Date:

Age at examination:

General aspects

Bodily habitus:

 Developmental level
 motor :
 cognitive:

Stature in proportion: yes/no

Measurements

P/SD

P/SD

weight			Ear length	AD AS		
length/height			Nipple distance			
OFC			Chest circumference			
spanwidth			Penile length			
US/LS			Testis volume	ri le		
Sitting height			Foot length	ri le		
ICD			Hand length	ri le		
OCD			Palml ength	ri le		
IPD			finger III length	ri le		
Palp. fissures						
Corneadiameter	OD OS					
fontanel						

HEAD

<u>General:</u>	form	micro/retrognathia
	forehead	mimics
	mid face	
<u>Eyes</u>	: position	form
	hypo/hypertelorism	tele/epicanthus
	blepharophimosis ri/le	ptosis ri/le
	microphthalmos ri/le	iris coloboma
	cornea	eye lids
<u>Ears</u>	eye colour	eye movements
	: position	fistula
	form	appendages
<u>Nose</u>	: form	philtrum
	choanae	
<u>Mouth</u>	: size	palate (uvula)
	lips	teeth
	tongue	gingiva
<u>Neck</u>	: webbing	hairline
	fistula	movements

TRUNK

<u>Thorax</u>	: form	heart
	mammae	lungs
	nipples	
<u>Abdomen</u>	: liver	spleen
	kidneys	hernia
	diastasis mm. recti	abdominal wall

Back : kyphosis/lordosis/scoliosis spina bifida
sacrale dimple anus

Genitalia : puberty stages (Tanner) A M P G
testis

LIMBS

Arms : proportions upper arm
muscle tone under arm
hands: syn/poly/clino/camptodactyly
palm creases

Legs : proportions upper leg
spiertonus lower leg
feet: syn/poly/clino/camptodactyly
pes cavus/planus

Hypermobility score: thumb to under arm 5th finger > 90°
elbows > 10°
knees > 10° hands to floor

Total/9

Contractures?

SKIN

Hair (incl.eyebrows, eyelashes) sweating
elasticity nails
pigment changes others
bullae/ichthyosis/hyperkeratosis
vascular abnormalities

NERVOUS SYSTEM

Summary**Preliminary conclusion and differential diagnosis****Additional investigations/management****Results of additional investigations** (with dates!)

biochemistry/clinical chemistry

cytogenetics

DNA

imaging

IQ

consultands

Appendix 8. Clinical features summary on subjects with positive result of subtelomeric rearrangement in both STD probe-kits (5 Subjects).

Clinical Features	Total	Percentage
Level of mental retardation		
Mild	3	60%
Moderate	2	40%
Height	2	40%
Short stature	2	40%
OFC	3	60%
Microcephaly	3	60%
Behaviour problems *)	1	20%
Autistic	1	20%
Aggressive	1	20%
Other features		
Cranial/Face *)	2	40%
Brachycephaly	1	20%
High Forehead	1	20%
Retrognathia	1	20%
Ears *)	3	60%
Low-Set	1	20%
Prominent	2	40%
Posteriorly rotated	2	40%
Prominent anti-helix	1	20%
Ocular region *)	5	100%
Ptosis	2	40%
Telecanthus	3	60%
Strabismus	2	40%
Downslanting palpebral fissure	1	20%
Deep-set eyes	1	20%
Hypertelorism	1	20%
Nose *)	3	60%
Flat nose	1	20%
Broad Nasal bridge	1	20%
Deviated columnella	1	20%
Depressed nasal bridge	1	20%
Broad base	1	20%
Mouth area *)	5	100%
Short philtrum	3	60%
Teeth overbite	1	20%

High arched palate	1	20%
Clinical Features	Total	Percentage
Cleft lip	1	20%
Downturned of mouth	1	20%
Thorax *)	2	40%
Pectus Excavatum	1	20%
Wide-spaced nipples	1	20%
Extremities *)	4	80%
Tapering fingers	1	20%
Clinodactily 5th finger	1	20%
Sandal gaps	1	20%
Cubitus valgus	1	20%
Short 5th finger	1	20%
Single palmar crease	1	20%
Other features *)	1	20%
Delayed speech	1	20%
Poor coordination	1	20%

*) One subject may have one or more abnormalities in this region